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THE RELATIONSHIP BETWEEN CATECHOL OESTROGEN AND BLADDER PATHOLOGIES IN HUMAN URINARY SCHISTOSOMIASIS

BY

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DECLARATION

This thesis is a research work undertaken by Ama Afrah in the Department of Animal Biology and Conservation Science, Department of Medical Microbiology, Department of Pathology and National Public Health, Reference Laboratory, Pathology without Borders Laboratory and Accra Technical University under the supervision of Professor Richard Kwasi Gyasi, Professor Patience Borkor Tetteh-Quarcoo, Dr. Bethel Kwansa-Bentum and Dr. Godfred Futagbi.

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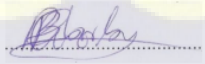

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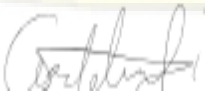

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DEDICATION

This thesis is to God, my parents Mr and Mrs (late) Donkor, Dr. Kwame Osei (husband) and parents in law Mr. and Dr. Mrs. (late) Owusu, Kelly Buabeng (son), Kwadwo Appau, Kyle Atobra Donkor nephew, my entire family and all study participants.

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LIST OF ABBREVIATIONS

ANOVA ……..……Analysis of variance

BP……..……. Base pairs

cDNA……Complementary Deoxyribonucleic acid

CE…..Catechol oestrogen

COMT…….……Catechol -O-methyltransferase

DAB…….……..Diaminobenzidine

DALY……...……..... Daily Adjusted Life Years

D…….……..……..Dalton

DNA…… Deoxyribonucleic acid

DPX…….…… Distyrene plasticizer xylene

EA……...……Eosin Azzure

ER……..Oestrogen receptor

FPR…….……..…False positive rate

HIV/AIDS…..Human immunodeficiency virus/acquired immune deficiency syndrome

HK……Hyperkeratosis

HRP…….……...…. Horse radish peroxide

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ID……..……..…..Infectious diseases

IRB……Institutional Review Board

K-SM……..…..…… Keratinizing squamous metaplasia

LMIC……...……..……low-middle income country

mRNA……...…Messenger ribonucleic acid

NCD……Non-communicable diseases

NK-SM……...……Non-keratinizing squamous metaplasia

NMIMR…… Noguchi Memorial Institute of Medical Research

NTD……Neglected tropical disease

OD…… ……..……..Optical density

Pap……..…..……..…..Papanicoulaou

PBS…….Phosphate Buffered saline

RBC…….……...Red Blood Cell

RPM……...……..…Revolutions per minute

SCC…..Squamous Cell Carcinoma

SEA……...……Schistosoma egg antigen

SHS……..…….Senior High School

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S. h……..…….*Schistosoma haematobium*

S. haematobium……...……*Schistosoma haematobium*

SM …….…….,,,....Squamous Metaplasia

SCC……..…Squamous Cell Carcinoma

TCC……. Transitional Cell Carcinoma

TPR……..……..…..True positive rate

UCC…….……....Urothelial Cell Carcinoma

UD ……..…..Urothelial Dysplasia

Vs ……...……...……...……Versus

WHO……..……..…..……World Health Organization

WHO-IACR……..……World Health Organization-International Agency for Cancer Research

X^2……..……...……..……..Chi square

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ABSTRACT

Bladder pathologies associated with urinary schistosomiasis begin in the subepithelial tissues before invading the epithelial tissues. Urine cytopathology although specific with an added advantage of non-invasive sampling, has a limitation because subepithelial bladder pathologies cannot be picked up. They do not exfoliate into the urine that is used for diagnosis or screening. *Schistosoma haematobium* egg antigen contains catechol oestrogen (CE) which is categorized as a class I carcinogen and soluble in urine. Archived bladder biopsies from the Department of Pathology, University of Ghana Medical School and urine samples from two *S. haematobium* endemic communities (Zenu and Weija) were used for the study. Both the community urine samples and archived tissue samples were analysed for the presence of *S. haematobium* ova using wet preparation and Haematoxylin and Eosin staining technique respectively. Again, smears were prepared from urine sediment, wet fixed and stained with Papanicoulaou staining technique for diagnosis of bladder pathologies by cytology. Tissue sections prepared from archived biopsies were stained with Haematoxylin and Eosin (H & E) for diagnosis of bladder pathologies by histopathology. Concentration of CE in urine was estimated using ELISA technique whilst immunocytochemistry was used to demonstrate biomarkers Ki67, cyclin D1 and HER2. Analysis of community samples showed that participants aged from 6-19 were 1.76 times more likely to have bladder pathologies with reference to those above 60 (OR=1.76, (95%CI; 1.039, 2.982) p=0.017) whereas males were 41% less likely to develop bladder pathologies than females (OR=0.410, (95%CI: 0.204, 0.825) P=0.0124). There was significant association between squamous cell carcinoma and the presence of *S. haematobium* ($\chi 2$=56.738, p<0.001). Patients diagnosed with squamous cell carcinoma were 6.8 times more likely to test positive for *S. haematobium* infection compared to those patients who tested negative [OR = 6.833, (96% CI: 3.944 to 11.839) p<0.001]. A highly significant correlation existed between ova counts and CE concentration in urine (r=0.4218, 95% CI: 0.3379 to 0.4991, p<0.001).

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Significant differences in CE concentration were also observed among the categories of bladder pathologies ($p < 0.001$).

Using urine samples, the Receiver Operating Characteristic (ROC) curves show that CE has an excellent predictive ability to discriminate bladder pathology patients from normal individuals and more sensitive than the other molecular markers such as Ki67, cyclin D1 and HER2. This study found a strong statistical correlation between some bladder pathologies (squamous cell carcinoma, urothelial dysplasia squamous metaplasia) and schistosomiasis infection. High concentration of CE was associated with *S. haematobium* infection and abnormally high expression of Ki67 is expressed in bladder pathology in endemic areas

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CHAPTER ONE

1.0 INTRODUCTION

1.1 Background to the Study

Schistosomiasis is a neglected parasitic tropical disease in terms of socioeconomic impact and public health importance (French *et al.*, 2018; Adenowo *et al.*, 2015; Fatimah *et al.*, 2015). Globally, over 205 million people persistently suffer from the disease with an annual mortality of 200, 000 people (Verjee, 2019). Schistosomiasis has devastating effect on development of children, pregnancy outcome and agriculture productivity, with most of the affected people living in poverty especially in sub-Saharan Africa (Rinaldo *et al.*, 2021). Ghana bears 15% of the burden of schistosomiasis in sub-Saharan Africa (Rollinson, 2009).

There has been prediction of increase in global prevalence rate of schistosomiasis which is primarily attributable to climate change, travel and leisure as well as outbreaks of wars but the countries likely to suffer most are the poor ones because of their inability to finance or sustain control programs (Vieira *et al.*, 2007; Mostafa, Sheweita, & O'Connor, 1999; Martens, 199).

Insensitive methods of diagnosis which use presence of ova in urine and stool as a hallmark of schistosomal infection have resulted in wrong data affecting policy formulation and implementation on the disease (Colley *et al.*, 2017). Inadequate data on genomic studies has resulted in wrong data on *Schistosoma* infection affecting formulation of current drugs and development of vaccine on the disease (Molehin *et al.*, 2016).

The five (5) different species of *Schistosoma* that infect human beings are *Schistosoma mansoni*, S*chistosoma haematobium*, *Schistosoma japonicum*, *Schistosoma mekongi* and *Schistosoma intercalatum* (Anyan *et al.*, 2019; Nacif-Pimenta *et al.*, 2019; Leão *et al.*, 2016). Persistent infection with these species may manifest as cancers due to gene mutation in humans (Saeed *et*

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al., 2019; Anderson *et al.*, 1992). *Schistosoma haematobium*, the causative agent of human urinary schistosomiasis is responsible for over 65% of all schistosomiasis worldwide and breeds throughout the year (Dent & King, 2007). There is a strong association between people suffering from urinary schistosomiasis and the risk of developing bladder cancer, especially in sub-Saharan Africa where less than 30% receive treatment (Aula *et al.*, 2021).

Children are more vulnerable to urinary schistosomiasis with resultant cognitive decline, malnutrition and anaemia (Brindley *et al.*, 2016; Victor A., 2014; Yirenya-Tawiah *et al.*, 2011Hodder *et al.*, 2000; Nkegbe E., 2010). Schistosoma egg antigen (SEA) contains catechol oestrogen (CE), a substrate for the production of catechol –O-methyltransferase (COMT). Catechol-O-methyltransferase is responsible for the breakdown of CE to form semiquinone with a genotoxic property damaging DNA with resultant cell cycle gene mutation and subsequent development of bladder pathologies in vulnerable people infected with *S. haematobium* (Giudice *et al.*, 2019; Yager, 2012). Persistent bladder pathologies can result in impairment of urinary function and eventual death of patient (Agbor *et al.*, 2016; Khan *et al.*, 2002). Therefore, CE in urinary schistosomiasis could be associated with bladder pathologies and induces abnormal cellular proliferation, which correlates positively with Ki67 expression, a nuclear proliferative protein (Botelho *et al.*, 2017; Mosli *et al.*, 2013).

1.2 Problem statement and study rationale

Bladder cancer ranks as the sixth most prevalent cancer globally, with its incidence and prevalence rising steadily, mostly followed by high morbidity and mortality (Batista *et al.*, 2020). Bladder cancer is a complex disease with several molecular and pathological pathways, thus reflecting different behaviours depending on the clinical staging of tumour and molecular type.

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A greater number of bladder cancer cases arises from epithelial cells and approximately 90% are urothelial tumours, with squamous and glandular-type tumours as less frequent histologic sub-types (Batista *et al.*, 2020). In 2018, approximately five hundred thousand people were diagnosed with bladder cancer and about two hundred thousand people died from it (Batista *et al.*, 2020). Overall, the mortality rate of bladder cancer in 2018 was 1.9 in 100,000 (Batista *et al.*, 2020). However, there are large variations in bladder cancer mortality, suggesting that some factors such as environmental could play a role in the aetiology of bladder cancer (Abol-Enein, 2008).

Many studies have shown that schistosomiasis is aetiologically related to the development of bladder cancer in individuals infected with *Schistosoma haematobium* (Zaghloul, 2012; Abol-Enein, 2008). The ova of the schistosome are deposited within the wall of the bladder, triggering an inflammatory response which continues over the lifespan of the infected person mostly, especially if the person is re-infected. Mostly associated with this inflammatory response is the conversion of the transitional epithelium to a squamous metaplastic epithelium, which has a much greater proliferative rate (Abol-Enein, 2008). Consequently, approximately 70% of people suffering from chronic schistosomiasis who have bladder cancer develop squamous cell carcinoma rather than transitional cell carcinoma (Johansson & Cohen, 1997). The incidence of urogenital schistosomiasis-associated squamous cell carcinoma is estimated in three to four cases per 100, 000 (Felix *et al.*, 2008). The mechanism by which schistosomiasis produces bladder cancer remains unknown, but two factors appear to be relevant. The first of these is the increased inflammatory and regenerative process in the bladder of these patients. In addition, the inflammatory process and exposure to environmental agents have the potential to generate genotoxic substances in the urine (Batista *et al.*, 2020).

When lots of schistosome ova fail to leave the body, they get lodged within interstitial tissues of different organs. Ova of schistosome trapped induce granulomata and may cause haematuria (Eissa

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et al., 2015). In view of that, eggs of *S. haematobium* are considered powerful biological carcinogens; hence it has been known to be implicated in the aetiology or pathogenesis of bladder cancer (Eissa *et al.*, 2015).

Prevalence rate of urinary schistosomiasis in Ghana has escalated post-independence (1963-2010) with some shorelines of the River Volta previously without any history or incidence of schistosomiasis rising to hyper-epidemic levels due to irrigated farming practices (Mone *et al.*, 2010; WHO, 1987). Many rivers such as Volta, Densu and some irrigation dams such as the one at Zenu are infested with cercariae of *S. haematobium* due to the abundance of *Bulinus* snail species serving as intermediate host for the parasites and conducive environment for breeding (Anyan *et al.*, 2019; Amoah *et al.*, 2018;; Rashid *et al.*, 2011). These *S. haematobium* endemic communities also lack health facilities that will treat the symptoms of urinary schistosomiasis caused by *S. haematobium*.

In *S. haematobium* endemic communities worldwide, the prevalence of bladder pathologies (several urothelial lesions) is high and preventive treatment of urinary schistosomiasis has resulted in a reduction in bladder pathologies suggesting an association between urinary schistosomiasis and bladder pathologies (Botelho *et al.*, 2018; Gouveia *et al.*, 2015; Botelho *et al.*, 2010). In Egypt as well as other countries where urinary schistosomiasis is endemic, over 25% of all reported cancer cases are *S. haematobium* associated bladder cancers (Onile *et al.*, 2016; Khaled, 2013; Elsebai, 1977). In Ghana, there has been demonstration of systemic involvement by schistosomiasis in surgical specimen at Korle-Bu Teaching Hospital manifesting as several of urothelial lesions (Der *et al.*, 2015).

Bladder pathologies due to urinary schistosomiasis start in the sub-epithelial tissues and later involve the epithelial tissue all of which can be diagnosed by histopathological method while cytological

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diagnosis is limited to the epithelium only (Zaghloul *et al.*, 2020; Tetteh-Quarcoo *et al.*, 2019). In cytology, among the epithelial lesions associated with urinary schistosomiasis are keratinizing squamous metaplasia (K-SM), non-keratinizing squamous metaplasia (NK-SM), hyperkeratosis, squamous cell carcinoma (SCC), urothelial dysplasia (UD) and occasional urothelial cell carcinoma (UCC) which is burdened with inconsistencies in grading worldwide (Bell *et al.*, 2020; Benelli *et al.*, 2018). An attempt has been made by the Paris System for Reporting Urinary Cytology of urothelial cells and the Bethesda System of Reporting Urine Cytology to standardize the reporting of urine cytology but to no avail. These epithelial cells exfoliate into urine to enable cytological analysis (Carvalho *et al.*, 2020; Sullivan *et al.*, 2010). They also provide an added advantage to the sampling procedure for cytology, in that it is non-invasive (Sullivan *et al.*, 2010; Tzanetou *et al.*, 2007). However, cytological diagnosis can miss bladder pathology of the sub-epithelium (connective tissues and muscle) in urinary schistosomiasis, therefore there is the need to use biomarkers to screen for those who are at risk of developing bladder pathology due to urinary schistosomiasis (Adebayo *et al.*, 2018; Shiff *et al.*, 2006).

Xenoestrogens have been implicated in cancers (Sahib M., 2019). The ova of *S. haematobium* contain catechol oestrogen (CE) hypothesized to be associated with bladder pathologies in urinary schistosomiasis (Smith *et al.*, 2006; Straub R., 2007). Catechol oestrogen contained in ova of *S. haematobium* has a genotoxic property resulting in cell cycle genes mutation in the urothelium. There is limited understanding of the disease mechanism. The parasite gene make up with its interaction with the host had not being studied. Hence the understanding of the disease mechanism is not understood. Inadequate data on genomic information affects the development of accurate diagnostic test to replace the existing with diagnostic pitfalls therefore delaying treatment or misdiagnosis. The existing drug is not optimal and needs to be improved but lack of data on

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genomic study hinders development of potential drug targets. Genomic study plays important role in monitoring drug resistance, but this is lacking. There is the urgent need for genomic study for development of vaccines and therapeutic drugs

These leads to persistence of urinary schistosomiasis with resultant bladder pathology. Mutation in the urothelium progressive bladder pathologies characterized by abnormally high cellular proliferation. Abnormally high cellular proliferation can be assessed using Ki67 expression in nuclei in urinary schistosomiasis (Teng *et al.*, 2015; Mosli *et al.*, 2013; Botelho *et al.*, 2009; Cavalieri & Rogan, 2006). Hence, CE can be used as a biomarker to screen for those who are at risk of developing bladder cancer in the future in *S. haematobium* endemic communities especially in the absence of epithelial bladder pathologies (Metwally *et al.*, 2011).

1.3 Objectives of the study

The objectives for the study were as follows:

1.3.1 Main Objective

The overall aim of the study was to determine the association between bladder pathologies in urinary schistosomiasis and catechol oestrogen.

1.3.1.1 Specific Objectives

The specific objectives of this study are to:

1. determine the prevalence of urinary schistosomiasis among patients' bladder biopsy samples and community urine samples.
2. determine prevalence of bladder pathologies among

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3. determine association between urinary schistosomiasis and bladder pathologies in patients' bladder biopsy samples and community urine samples

4. determine the relationship between catechol oestrogen levels in urine and the quantity of *Schistosoma haematobium* ova

5. assess correlation between catechol oestrogen and bladder pathology

6. assess the diagnostic performance of catechol oestrogen, cyclin D1, HER2 and Ki67 as markers in urine to diagnose bladder pathologies.

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CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Brief history about schistosomiasis

Theodor Bilharz, a German pathologist was the first person to give a vivid description of the *S. haematobium* parasite in 1851 (Santos *et al.*, 2021). During the post-mortem examinations done in Cairo on Egyptian soldiers, Bilharz described a putative parasite responsible for injuries in a letter addressed to his mentor in Germany appointing them to the genus *Distomum*. Thus, *Schistosoma* was firstly termed as *Distomum haematobium*. Bilharz also described the emergence of embryos from the eggs in the bladder and subsequent passage in urine to freshwater. David Friedrich Weinland in 1858 was the person who proposed the name *Schistosoma* due to the morphology of the male worms and thus was adopted by the International Commission on Zoological Nomenclature (Di *et al.*, 2018). Weinland observed significant differences between the newly discovered parasite and the species belonging to *Distomum*, hence the new genus. *Schistosoma* is derived from two Greek words '*schistos'* meaning split and '*soma'* meaning body. To honour Theodor Bilharz, Cobbold proposed the change of the genus *Schistosoma* for Bilharzia in 1859. The name schistosomiasis predominates currently notwithstanding the frequent references to the disease as bilharziasis in French and Portuguese medical literature. During the same year, Harley and Cobbold stated that the human infection occurred percutaneously. Cobbold also observed that schistosomes were not restricted to human beings when he found '*Bilharzia magna'* in a West African monkey. Later on, definitive hosts of schistosomes were discovered in other primates, ruminants, rodents and cattle (Di *et al.*, 2018).

Patrick Manson (the 'father of tropical medicine') in 1902, suggested the existence of two distinct species of *Schistosoma* and a few years later, in 1908, schistosomiasis was detected for the first time in Brazil. The contribution of Piraja da Silva was very key in identifying the two distinct species: *S.*

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haematobium and *S. mansoni* (Katz, 2008). The infection with these two species was conclusively recognized by Leiper in 1915 who reported that the lifecycle of *Schistosoma* included a freshwater snail as intermediate host (Santos *et al.*, 2021).

During Napoleon's invasion of Egypt (1799 – 1801), *S. haematobium* infected the French troops stationed in Egypt. The French military doctors ascribed the haematuria to the sweating and climate of Egypt, or as the 'revenge of the Pharaohs'.

2.1.1 Evidence of schistosomiasis in ancient times

Schistosomiasis is an ancient disease, but due to its unusual lifecycle, it is noted as 'the most important water-based disease from a global public health perspective' signifying the fundamental role of water for its spread (Steinmann *et al.*, 2006). Studies conducted on skeletons of humans discovered in the vicinity of Tell Zeidan, an initial settlement of farmers in Northern Syria (5800 – 4000 BC), demonstrated the evidence of a terminal spined schistosome from the pelvic sediment of the skeletal remains (Anastasiou *et al.*, 2014). Subsequently, it was believed that schistosomiasis travelled to Egypt as a result of the importation of monkeys and slaves during the reign of the fifth dynasty of pharaohs (Di *et al.*, 2018).

Ever since the dawn of the Egyptian civilization, parasitic flatworms *Schistosoma* have lurked in the Nile river (Sarant, 2017). The 'a-a-a disease', a possible ancient name for schistosomiasis, has been mentioned in several Egyptian papyri from as early as 1500 BC, and described as a disease characterized by discharge from the penis. Nevertheless, there is no assurance that it was certainly schistosomiasis, due to the multiple differential diagnoses (Di *et al.*, 2018). Nevertheless, the ancient

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Egyptians were advised to avoid contact with polluted water (Sarant L., 2017). Several ancient anecdotes reported conditions similar to schistosomiasis. For example, evidence in ancient Egyptian medical papyri or Assyrian medical texts described signs and symptoms that closely resemble schistosomiasis. Similarly, some passages in the Bible describe an epidemic (described as a 'curse') that has been hypothesized to be correlated with schistosomiasis spread in Mesopotamia (Di *et al.*, 2018).

2.1.2 Evidence of schistosomiasis in the 'modern era'

An Italian Physician and Botanist Prospero Alpine (1553 – 1616) was the first to notice a high incidence of haematuria in Egypt, two centuries before other European researchers. A French doctor by name Renault in 1798, described Egypt as the only country where men menstruate (Sarant, 2017). He described an obstinate haematuria affecting many of the French soldiers in Egypt. The unknown disease presented with pain in the bladder, extending along the urethra (Sarant, 2017).

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2.2. Schistosomiasis as a public health problem

Schistosomiasis commonly known as bilharziasis was originally zoonotic, infecting non-human primates and sustenance of the disease has been possible due to diphasic life cycle in human and snail host (Catalano *et al.*, 2018; Standley *et al.*, 2012). It is the devastating tropical and sub-tropical water-borne parasitic disease in terms of public health importance due to the presence of fresh water bodies and conducive atmosphere in endemic communities (Kaliyaperumal *et al.*, 2017; Forstinus & Ikechukwu, 2016; Adenowo *et al.*, 2015; Utzinger *et al.*, 2009). Human schistosomiasis caused by trematode of the genus *Schistosoma*, forms a third of all parasitic infections worldwide (Colley *et al.*, 2014). There are variations in manifestations depending on the species the *Schistosoma* causing the disease (Costain *et al.*, 2018; Downs *et al.*, 2017; Kaliyaperumal *et al.*, 2017; Fatimah *et al.*, 2015; Lacerda-Queiroz *et al.*, 2013). The disease is a major cause of morbidity affecting the lungs, liver, gastrointestinal tract and the urinary system depending on the species and the organs it infects (Tang *et al.*, 2012; Wu & Halim, 2000). However, in some rare cases, ectopic infections can occur, posing a threat to diagnoses, since the species may not be seen in the usual specimens for examination (Nunes *et al.*, 2013; Anyan *et al.*, 2019; Imai *et al.*, 2011; Wan *et al.*, 2009). When schistosomiasis is not treated, it causes deformities in affected organs which impairs organ function and can evolve into malignancies (Arora *et al.*, 2019; Gouveia *et al.*, 2019; Brindley *et al.*, 2016; Brand, 1979;).

Travel, immigration, tourism, trade, peace keeping missions among other activities have made a tropical disease like schistosomiasis, a global one. In the United States of America and other countries, there have been reported cases of ectopic *S. haematobium* infection manifesting as cranial schistosomiasis (Imai *et al.* 2011; Dent *et al.*, 2007). Schistosomiasis transmission is reported in over 75 countries but only a little above 30 countries receive treatment with praziquantel (Botelho,

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2017; Kaliyaperumal *et al.*, 2017). Over 200 million people required preventive treatment in 2012 of which over 70% are in sub-Saharan Africa due to the presence of fresh water bodies (Anyan *et al.*, 2019).

Although some successes have been achieved in controlling schistosomiasis in some endemic communities using Mass Drug Administration (MDA), over 200 hundred million people throughout the world still suffer from schistosomiasis with over 80% of the people suffering from the disease being in developing countries (Stothard *et al.*, 2017; Adenowo *et al.*, 2015; Chitsulo *et al.*, 2000).

In Japan and Tunisia, schistosomiasis has been eliminated using the one-health approach by targeting the various stages of development of the parasite's diphasic life cycle whilst Morocco an d Caribbean are on the way to elimination of the disease (Adenowo *et al.*, 2015; Utzinger *et al.*, 2009).

Persistence in global prevalence rate (over 200 million people) of schistosomiasis is partly attributable to travel and leisure, whereas outbreaks of wars are predicted to raise the cases of schistosomiasis to hyperendemic levels but the countries likely to suffer most are the poor and developing ones because of their inability to finance and sustain control programs (Leão *et al.*, 2016; Landouré *et al.*, 2012). This will subsequently affect elimination or eradication of the disease because funders of such programs dictate what must be done that may not help the affected countries. In a similar situation, schistosomiasis control program in Uganda where infection rate of the study population was over 60%, the target of the funders was to treat 20% of the disease leaving 80% residual; a recipe for re-emergence of the disease (Loewenberg S,, 2010).

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2.2.1. Schistosomiasis as a Neglected Tropical Disease

Neglected Tropical Diseases (NTDs) constitute a group of chronic, disabling and disfiguring disease and conditions that happen most usually in situations of severe poverty, particularly among the rural poor and some disadvantaged urban populations (Hotez *et al.*, 2007). The concept of NTDs as a group of chronic and debilitating parasitic and related infections emerged in 2004-2005 (Botelho & Sousa, 2014). These NTDs represent a group of bacterial, parasitic, viral and fungal infections that are prevalent in many of the tropical countries of the developing world where poverty is very common (Mitra & Mawson, 2017). They are neglected because they are almost absent from the global health agenda.

More than a billion of the world's population especially those in developing countries are infected with one or more of the NTDs (Mitra & Mawson, 2017). Several studies have shown that the NTDs are widespread among the poor living in sub-Saharan Africa (Brooker *et al.*, 2006; Molyneux *et al.*, 2005) with the commonest NTDs such as soil-transmitted helminth infections and schistosomiasis affecting many people (Fenwick, 2006).

Approximately 250 million people are afflicted with schistosomiasis and 85% of them reside in sub-Saharan Africa (Sacolo *et al.*, 2018). The disease is endemic in over 78 resource-constrained countries, and is one of the pointers of poverty because it is often ever-present among the poor people of the world mostly in sub-Saharan Africa (Karunamoorthi *et al.*, 2018). Approximately, 76% of the population in sub-Saharan Africa live near rivers, lakes and other water bodies contaminated with snail intermediate hosts (Hotez & Kamath, 2009; Steinmann *et al.*, 2006), most especially populations residing near dam reservoirs who are at the greatest risk of infection with *Schistosoma* (Steinmann *et al.*, 2006). There are several examples from sub-Saharan

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African countries where cases of schistosomiasis had considerably risen due to irrigation projects because dam bocks migration of (Sokolow *et al.*, 2017). Notwithstanding its tremendous public health significance, and its well-established associations with HIV/AIDS and cancer, *Schistosoma haematobium* has been considered 'the neglected schistosome' (Brindley & Hotez, 2013).

2.2.2. Global burden of schistosomiasis

The Global Burden of Disease (GBD) study aims to "determine the cumulative global disability that is attributable to diverse diseases, the knowledge of which is essential in targeting public health responses, prioritising research and guiding research" (King & Galvani, 2018). The 2016 GBD study suggested that of all the 328 diseases which were considered, schistosomiasis was observed to indicate the most noticeable decline in age-standardized years lived with disability (LYD) between 2006 and 2016 (Stolk *et al.*, 2016). Globally, schistosomiasis has been ranked in the top ten for YLDs in six sub-Saharan African nations, however, recently it has been reported to account for only 1.496 million YLD (King & Galvani, 2018). From the 2010 GBD study, NTDs were responsible for 26.06 million disability-adjusted life years (DALYs) (95% confidence interval: 20.30, 35.12) (Hotez *et al.*, 2014). The burden of NTDs is heavily concentrated in low and middle-income countries (Stolk *et al.*, 2016) and NTDs are often assumed to be concentrated in the poorest populations (Houweling *et al.*, 2016). The scale and trend of the disease burden in sub-Saharan Africa varies considerably, with poverty stricken and marginalized communities the worst affected (Houweling *et al.*, 2016). These people have low socio-economic status with limited access to clean water and improved sanitation facilities. Acute schistosomiasis also known as Katayama fever, has been reported to increase the mortality rate by up to 25% (Verjee, 2019; Ross *et al.*, 2007). Few people in the developed countries

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have adequate knowledge about NTDs but, taken together these diseases have a higher health burden than malaria, tuberculosis and HIV/AIDS (Hotez *et al.*, 2014).

Schistosomiasis ranks second only to malaria as the most common parasitic disease and is the most deadly NTD (Karunamoorthi *et al.*, 2018). Annually, schistosomiasis causes 280,000 deaths in developing countries of Africa, with an approximately 3.3 million disability-adjusted life years are lost (Hotez *et al.*, 2014). Schistosomiasis can lead to severe difficulties including anaemia, nutritional troubles, impaired cognitive capabilities and irreparable effects in old age such as cirrhosis and cancer (Linsuke *et al.*, 2014). The burden of schistosomiasis depends on oviposition in tissues which affects organ function due to organ deformities. Due to this, Daily Adjusted Life Years (DALY) of schistosomiasis is 10.4 by World Health Organization (WHO) (Adebayo *et al.*, 2018; French *et al.*, 2018; Colley *et al.*, 2014).

Use of praziquantel kills only mature worms of *Schistosoma* species leaving immature worms and ova, another setback in control programs (Strehlenert *et al.*, 2015). Of the more than 17 million people that were treated for schistosomiasis globally, over 55% were from sub-Saharan region in the year 2008 (Adenowo *et al.*, 2015). The output of work in adults suffering from Schistosomiasis is drastically reduced due to the burden of the disease as a result of high quantity of eggs. Productivity may then be reduced leading to economic loss and redundancy, compounding the already existing unemployment challenges (Haque, 2007; Oliver, 1974). Close proximity of households to contaminated water bodies has also contributed to persistent or recurrent infection because it increases one's frequency of contact with contaminated water containing cercariae, the infective form of the parasite (Adenowo *et al.*, 2015; Ahmed *et al.*, 2014).

Urinary schistosomiasis has adverse effect on health and economic implication (Aboagye & Edoh, 2009). Ghana has a nationwide prevalence of over 20% and a focal prevalence of over 50%. A

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parasitological survey prevalence is highest at the southern part of Ghana (Figure 1). Blood in urine nationwide survey revealed a prevalence of over 50%. Most of endemic communities in Northern Ghana have prevalence of *S. haematobium* between 1-9.9% (Global Atlas of Helminthes Infection, 2023).

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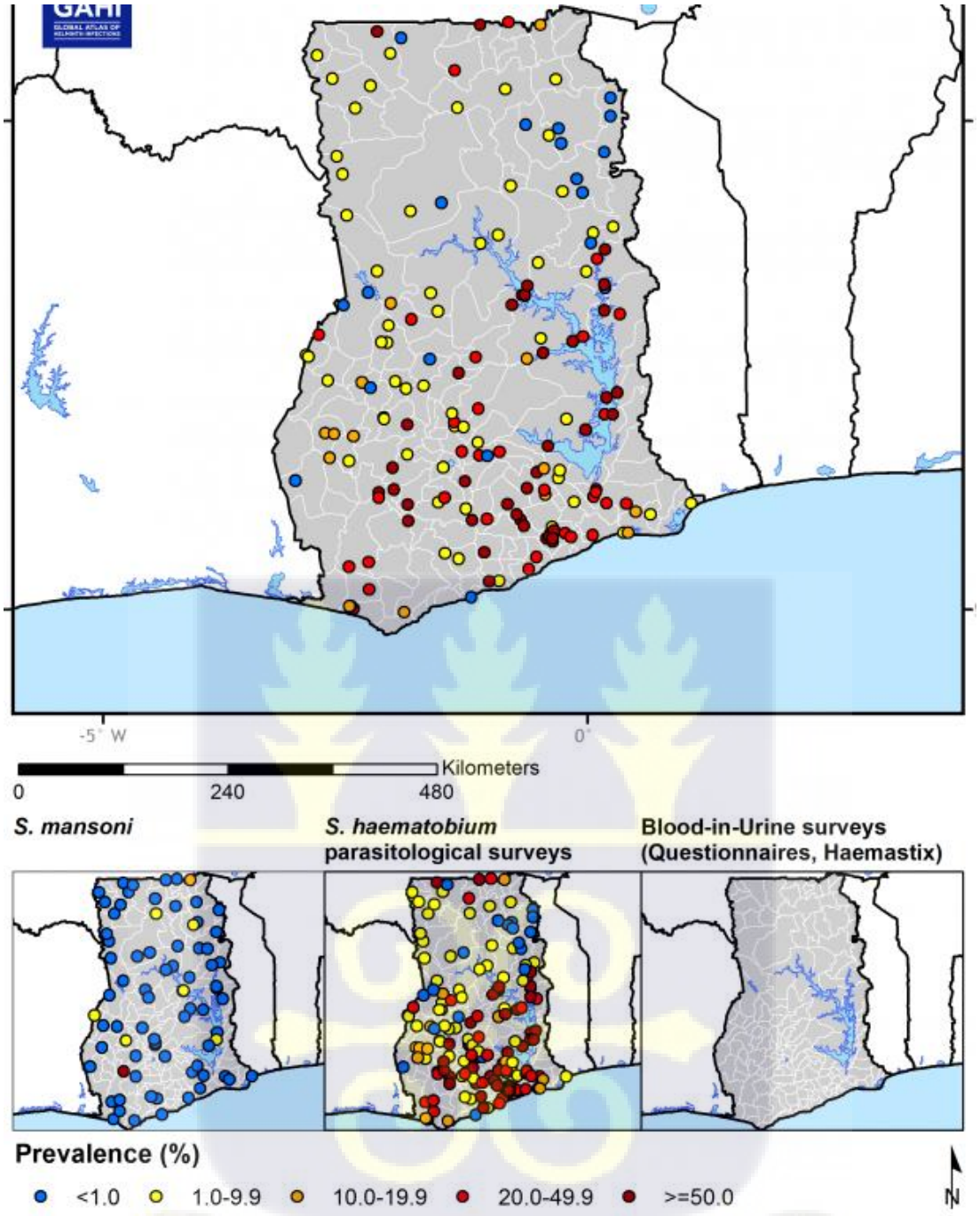

Figure 1: Distribution of schistosomiasis in Ghana

Source: Global Atlas of Helminthes Infection (2023).

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2.3. The biology of the Schistosome parasites

The six species that are pathologic to humans are *Schistosoma mansoni, Schistosoma intercalatum, Schistosoma japonicum, Schistosoma guineensis, Schistosoma mekongi* and *Schistosoma haematobium* (Boissier *et al.*, 2019; Nelwan, 2019). Humans are the definitive host in which the species *S. haematobium* that lives in venous plexus close to the bladder cause urinary schistosomiasis, whilst the others live in the mesenteric system and cause intestinal schistosomiasis (Nation *et al.*, 2020). The female *Schistosoma* lay about 2500 eggs in a day which are excreted by humans in urine in the case of *S. haematobium* mwhilst the eggs of the rest (*S. mansoni, S. intercalatum, Schistosoma japonicum, S. guineensis, S. mekongi)* are excreted through faeces (Schwartz & Fallon, 2018). Schistosomiasis is caused by accumulation of eggs that were not excreted in urine or faeces (Schwartz & Fallon, 2018; Gray *et al.*, 2011). The eggs serve as pathogens responsible for contaminating the environment especially water bodies. Fresh water snails serve as intermediate host in which the life cycle is completed. Infection with *Schistosoma* species occur when the unbroken skin is penetrated by cercariae. The eggs that are trapped in organs such as liver, intestines and bladder depending on the species. A few people develop acute inflammation in the initial phase of infection whilst most symptoms of schistosomiasis appear several months after infection. Manifestations of intestinal schistosomiasis include bloody diarrhoea, abdominal pain, fever and hepatomegaly (Nigo *et al.*, 2021; Carbonell *et al.*, 2021). Symptoms of urinary schistosomiasis include haematuria and dysuria. In persistent and chronic infections, urinary schistosomiasis can result in bladder pathologies including bladder cancer whilst intestinal schistosomiasis can result in liver cancers. Children are the most vulnerable to developing schistosomiasis with resultant anaemia, malnutrition and defunct cognitive function that prevent them developing their full potential (Ezeamama *et al.*, 2018; Osakunor *et al.*, 2018).

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Rare cases of *S. haematobium* infection, there is oviposition of ova in brain resulting in cranial schistosomiasis (Imai *et al.*, 2011). Diagnosis of urinary schistosomiasis is by seeing egg in urine deposit whilst diagnosis of intestinal schistosomiasis is by seeing in faeces (Ojo *et al.*, 2021).

Schistosomiasis transmission is reported in over 78 countries worldwide. Urinary contamination of freshwater with *S. haematobium* ova hatch into miracidia (Figure 2). The miracidia develop into cercariae, the form of *Schistosoma* which penetrate the *Bulinus* snail, the intermediate host's tissue (Grimes *et al.*, 2015). The miracidia develop into sporocysts and eventually evolve into cercariae in the snail host. The cercariae migrate from snail and actively swim in water. Cercariae the infective forms penetrate the unbroken skin of the definitive host (human) and shed its tail in the process to form schistosomula. The schistosomula enters blood circulation of definitive host entering portal blood circulation in liver and develop into adult worms. Male and female adult worms pair and migrate to venous plexus of bladder (Nation *et al.*, 2020). The cercariae migrate to the sub-epithelial tissues and eventually to the epithelial tissues of bladder, infected individuals pass urine into freshwater body and life cycle (Figure 2) continues.

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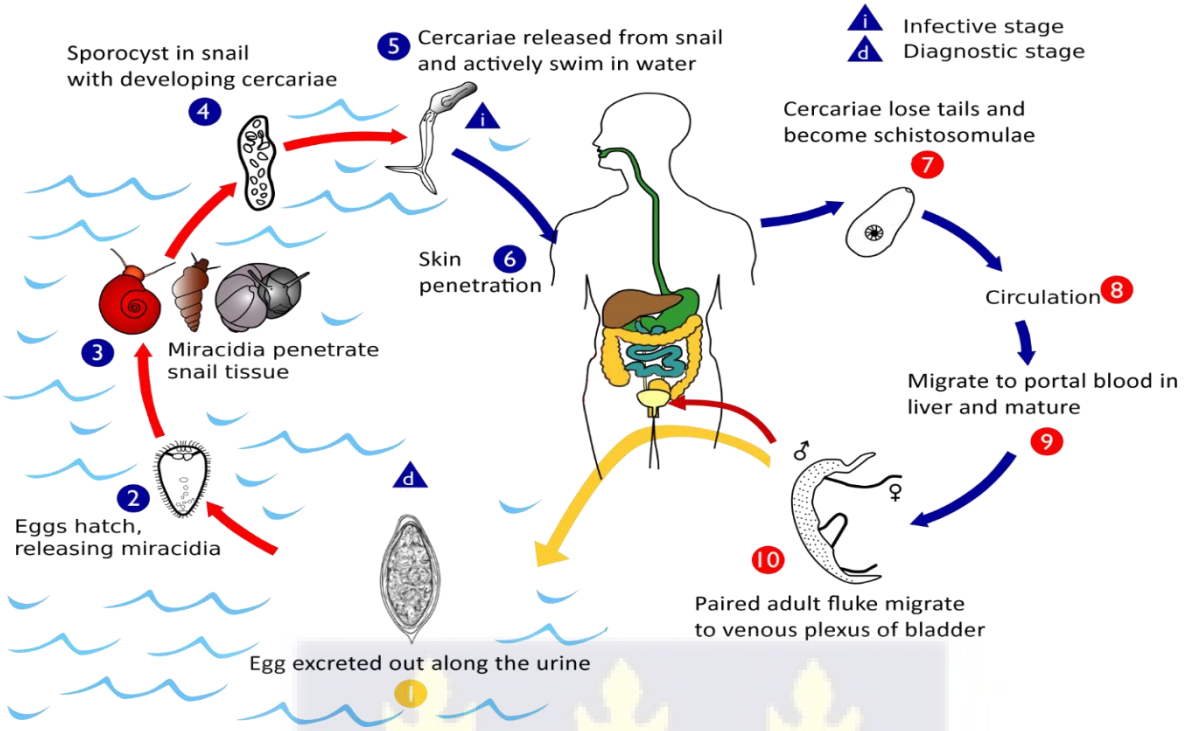

Figure 2: Life cycle of *Schistosoma* spp.

Source: Centre for Disease Control and Prevention (n.d.)

Unlike other water borne diseases where infection is through drinking contaminated water, with provision of potable water infection rate of *S. haematobium* is reduced drastically. *S. haematobium* infection is linked with frequency of contact with contaminated fresh water, routine agriculture practices, domestic and recreational activities (Forstinus & Ikechukwu, 2016; Secor, 2014). In *Schistosoma* endemic communities where there is lack of potable water, the people have no choice but to use the contaminated fresh water for domestic chores although most community members are not ignorant about the contamination of the water. The proximity of the fresh water to their homes makes it more convenient as their source of water than walking some distance to fetch potable water. Provision of potable water reduces contact of vulnerable population with contaminated fresh water

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even though variation in culture, socioeconomic status and other environmental factors could still influence transmission (Molehin, 2020; Nhidza *et al.*, 2017; Grimes *et al.*, 2015).

The presence of suitable intermediate snail host and favourable environmental conditions as well as climatic change in a community makes it endemic to *S. haematobium* if there are vulnerable population. *Bulinus* and *Biomphalaria* species are the snail hosts for *S. haematobium* the aetiologic agent of urinary schistosomiasis and *Schistosoma mansoni* the aetiologic agent of enteric schistosomiasis respectively (Amoah *et al.*, 2018; Abou-El-Naga, 2013). Some communities have both snail species hence both urinary schistosomiasis and enteric schistosomiasis are prevalent. The risk of co-morbidity is that the 2 different species of *Schistosoma* interact but the mechanism of interaction and the outcome of any morbidity is not known (Nyarko *et al.*, 2018; Knowles *et al.*, 2015).

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2.4. Prevention and control of schistosomiasis

Schistosomiasis is a parasitic infection that has co-evolved with human development and civilization (Di *et al.*, 2018). Schistosomiasis is the most important helminthiasis worldwide in terms of morbidity and mortality (Santos *et al.*, 2021). Most of the infections occur in Africa, which about two-thirds are caused by *Schistosoma haematobium*. The infection with *S. haematobium* is considered carcinogenic leading to squamous cell carcinoma and urothelial carcinoma of the urinary bladder (Zaghloul *et al.*, 2020).

The best way to control most infectious diseases is through vaccination but unfortunately there is no vaccine available presently because the species schistosomiasis has over 200 surface antigens for which only 25 have been successfully attenuated for successful vaccine production. To prevent transmission of schistosomiasis in endemic communities one must avoid wading in contaminated fresh water. Swimming pools and fresh water can be chlorinated. Water suspected to contain cercariae or stored in a tank should be boiled at 100^0C and cooled before using to bath. Towel exposure to contaminated water must be vigorously dried before used. In case of incidental contact with contaminated fresh, one must get preventive treatment with praziquantel. Travel and leisure to endemic communities by tourist from non-endemic countries should be followed up with testing and treatment. Chemicals can be used to eliminate snail hosts but other species are endangered.

Control programs target periodic treatment of large population in endemic communities using mass drug administration of praziquantel to an entire endemic community with special attention to school children who are most vulnerable to schistosomiasis accompanied by reduced cognitive function that may result in dropping out of school. According to WHO, in the year 2019, out of over 230 million people that needed treatment for schistosomiasis, a little over 100 million people received treatment

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for the disease. Provision of toilet facilities, potable water, change in job and different recreational grounds other than contaminated fresh water could be useful approach in reducing *S. haematobium* transmission (Forstinus & Ikechukwu, 2016; Secor, 2014).

Vaccination is the most cost-effective approach to control of infectious diseases like schistosomiasis and prevent premature deaths of billions of children in several years in low and middle-income countries but unfortunately there is no vaccination against schistosomiasis and treatment relies on only one drug, praziquantel which kills adult worms but do not reverse lesions or deformities caused by the parasite (Molehin, 2020; Siddiqui *et al.*, 2011). One of the major setbacks to development of effective vaccine is that over 200 antigens have been identified but only 25 of them, offered protection against the disease (Coulibaly *et al.*, 2018; Tebeje *et al.*, 2016; Siddiqui *et al.*, 2011). Also, the surface molecule of *Schistosoma* species keep evolving therefore praziquantel resistant *Schistosoma* could develop calling for an urgent need to develop vaccine against *Schistosoma* (McManus *et al.*, 2020; Tetteh-Quarcoo *et al.*, 2020).

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2.5 Setbacks in control programs of urinary schistosomiasis

Access to healthcare is a pre-requisite for good health and fostering socioeconomic development, however most urinary schistosomiasis endemic communities in Ghana have no healthcare facility or have marginalized healthcare system such that early *S. haematobium* infection that is treatable results in chronic infection subsequent development of bladder pathologies and eventually death in vulnerable population (Sulemana & Dinye, 2014; Lake *et al.*, 2013). Despite active control as well as elimination programmes, the prevalence of urinary schistosomiasis is still high but for these programmes to be successful, accurate data on the disease is needed to inform decision making (Claude *et al.*, 2019; Deribe *et al.*, 2011; Nkegbe, 2010).

The diphasic life cycle of the parasite which is aquatic in intermediate host (*Bulinus* spp.) and terrestrial in definitive host must be considered in eradication strategies (Nelwan, 2019; Nhidza *et al.*, 2017; Abou-El-Naga, 2013). Effective eradication of urinary schistosomiasis must target fresh water snails that serve as intermediate host but breaking the life cycle by targeting just a developmental stage can result in new life cycle since the parasite may find a convenient host (Haggerty *et al.*, 2020). New life cycle requires funding for new research and new medication. Fresh water keeps decreasing in quality over the years due to poor sanitation. Sanitation, water and hygiene education plays important role in urinary schistosomiasis control programs (Grimes *et al.*, 2015). Poor sanitation is a major risk factor in transmission of the infective form of *S. haematobium* cercariae (Lamberton *et al.*, 2017).

Some setbacks in control of urinary schistosomiasis are with difficulty in diagnosis and treatment failure. The use of light microscopy can be insensitive at times, and subsequently affects accuracy of data used by the control program (Coulibaly *et al.*, 2016; Ephraim *et al.*, 2015). The choice of sample

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used in diagnosis depends on the type of species of *Schistosoma* causing the infection. Diagnosis of urinary schistosomiasis is based mainly on the presence of ova in urine by light microscopy but the sensitivity of this method of diagnosis is low since the ova are shed periodically and may be missed at the time of sample collection (Weerakoon *et al.,* 2015). Secondly in fresh infections, when the number of parasites is not quite significant, ova produced may be just few, so parasites are likely to be missed when diagnosed using light microscopy. In such situations serological or molecular diagnosis is most appropriate (Aryeetey *et al.*, 2013). Another setback is that use of praziquantel only kill mature worms leaving immature worms and ova. This coupled with the attitude of re-visiting contaminated water in endemic communities results in re-infection (Tetteh-Quarcoo *et al.,* 2020; Lamberton *et al.,* 2017; Strehlenert *et al.,* 2015).

Urinary schistosomiasis has adverse effect on health and economic implication on communities in which *S. haematobium* is endemic (Aboagye *et al.,* 2009). The output of work of adults suffering from urinary schistosomiasis is drastically reduced due to burden of the ova that reduces quality of life. Since productivity is reduced, the employer loses revenue because the output of employee with schistosomiasis does not reflect salary (Audibert *et al.,* 1998). This can lead to redundancy compounding the already existing unemployment problem. Children with urinary schistosomiasis may drop out of school because cognitive function is reduced and also due to the burden of the disease (Nkegbe, 2010). Lack of sanitation facilities such as toilet facilities results in open defecation and urination from infected individual into water bodies that are slow moving or stagnated (Kulinkina *et al.,* 2018). This attitude as well as praziquantel resistance *Schistosoma* species resulting in reinfection (Tetteh-Quarcoo *et al.,* 2020; Claude *et al.,* 2019; Kavana, 2018).

In some situations, the presence of suitable intermediate snail host and favourable environmental conditions as well as climatic change in a community makes it suitable for endemicity if there are

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vulnerable people (Adenowo *et al.*, 2015). *Bulinus* and *Biomphalaria* species are the snail host for *S. haematobium* the aetiologic agent of urinary schistosomiasis and *S. mansoni* the aetiologic agent of enteric schistosomiasis respectively (Haggerty *et al.*, 2020). The availability of snail hosts depends on favourable environmental conditions such as fresh water temperature and abundance of aquatic vegetation at the shorelines of contaminated fresh-water bodies which serve as food, habitat, repository for snail eggs and protection for snails from being washed ashore by water current (Rabone *et al.*, 2019; Yirenya-Tawiah *et al.*, 2011). The shorelines of water bodies in some endemic communities have both snail species hence both urinary schistosomiasis and enteric schistosomiasis are prevalent.

Poor sanitation is a major risk factor in transmission of the cercariae, infective form of *Schistosoma* species (Boelee *et al.*, 2006). The quality of fresh water that make it safe for domestic use keep decreasing over the years due to inadequate sanitation facilities or lack of it. Education in the use of appropriate sanitation facilities will therefore plays important role in urinary schistosomiasis control programs (Forstinus *et al.*, 2016). For complete elimination and effective control of urinary schistosomiasis, the diphasic life cycle of the parasite which is aquatic in intermediate host (*Bulinus* species) and terrestrial in definitive host must be considered (Boelee *et al.*, 2006). For sustainable elimination and eradication of schistosomiasis, combination of strategies that take into consideration the complete life cycle of the *Schistosoma* species is recommended as was the case in Japan and Morocco (Nelwan, 2019; Adenowo *et al.*, 2015; Grimes *et al.*, 2015). The persistence in global prevalence rate of schistosomiasis is primarily attributed to climatic change, travel and leisure as well as outbreaks of wars but the countries likely to suffer most are the poor countries because of their inability to finance and sustain control programs (Vieira *et al.*, 2007; Mostafa *et al.*, 1999; Martens, 1995). Inability of affected countries to finance local control programs affect elimination or

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eradication of the schistosomiasis. In a UK/US sponsored schistosomiasis control program in Uganda where infection rate in Kampala was over 60% of the study population using Mass Drug Administration(MDA), the target of the funders was to treat just 20% of people with schistosomiasis leaving 80% residual which is a recipe for re-emergence of the disease (Loewenberg, 2010). Some livestock such as cows are infected by *S. japonicum* so their droppings can contaminate fresh water in non-endemic communities. Presence of suitable host in fresh water can result in these communities becoming endemic. Persistent or chronic schistosomiasis is associated with several pathologies depending on the species. Chronic urinary schistosomiasis is associated with bladder pathologies but rarely has it been studied in Ghana.

Schistosomiasis is a major ongoing public health issue particularly in sub-Saharan African countries. *S. haematobium* is the most prevalent species in sub-Saharan Africa with an estimated 112 million individuals infected (Aula *et al.*, 2021). Nearly 71 million individuals experience haematuria, fifty percent of which have dysuria, and around 18 million people infected people suffer from urinary bladder pathology yearly (Aula *et al.*, 2021). Currently, the highest schistosomiasis prevalence globally is held by Nigeria (Dawaki *et al.*, 2015). The two commonest species, *S. haematobium* and *S. mansoni* are prevalent in Ghana (Anyan *et al.*, 2020; Cunningham *et al.*, 2020; Gyasi *et al.*, 2019). A recent longitudinal study in Ghana involving 2623 participants including pre-school aged children and adults recorded an overall prevalence of 44.24% for *S. mansoni* and 11.9% for *S. haematobium*, with the highest prevalence recorded among school-aged children (Cunningham *et al.*, 2020). In recent times, there have been significant progress with respect to the reduction in the number of new cases of schistosomiasis and its associated morbidity significantly.

A major challenge to control efforts is the critical lack of accurate data especially from a number of sub-Saharan African nations, on the prevalence, intensity and epidemiology of schistosomiasis (Aula

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et al., 2021). This might be due to inadequate or non-existence of proper surveillance mechanisms and monitoring of schistosomiasis leading to the inability to predict transmission routes and hotspots (Kalinda *et al.*, 2020; Sacolo *et al.*, 2018).

2.5.1 The convergence of non-communicable disease (NCD) and infectious disease (ID)

In today's globalized world, rapid unplanned urbanization, improvement in agriculture especially in rural communities and very powerful companies like tobacco companies are linked with changes in behaviour that highly increase the risk of chronic non-communicable diseases (Oni & Unwin, 2015). These behavioural changes include switch to poorer healthy diet, reduced exercises, and decrease in tobacco and alcohol consumption. These have been identified as key risk factors for the increase in NCDs (Oni & Unwin, 2015). Low- and middle-income countries (LMICs) signify the nexus of NCD and ID challenges (Remais *et al.*, 2013). Many LMICs of the world are undergoing rapid changes associated with developing high rates of NCDs while concomitantly battling high levels of certain infectious diseases including malaria and HIV/AIDS (Oni & Unwin, 2015). Evidence is mounting that an individual's NCD risk status may put the individual at even higher risk for key IDs that are very common in LMICs (Remais *et al.*, 2013). The convergence of NCDs and IDs in LMICs poses novel challenges and novel opportunities to merit changes in policy and research in dealing with this convergence. Many LMICs have significant dual burdens of NCDs such as cancer and diabetes and IDs including malaria, TB and HIV/AIDS (Remais *et al.*, 2013; Smith & Ezzati, 2005). NCDs and IDs both have similar features such as long-term care needs and diseases occurring in similar high-risk populations. Rural-urban migrants, who are very common in LMICs represent one of such high-

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risk individuals for both ID and NCD (Gong *et al.*, 2012). A very pivotal study from China in relation to chronic disease among Chinese migrant population revealed that as people from the rural areas migrate to the urban areas, they show rise in blood pressure on par with their new urban dwellers and markedly higher than the people they left behind in the rural areas (He *et al.*, 1991). So, as China's enormous migrant population moves from the rural areas to the urban areas, there are changes in their lifestyles and diets leading to a range of risk factors indicative of NCDs (Gong *et al.*, 2012; He *et al.*, 1991). This rural-urban migrants also carry traditional IDs including parasites, at levels higher than their rural home communities and this can put these migrants at increased risk of infections like TB (Jia *et al.*, 2008). There are also notable direct interactions including association with certain cancers and IDs and also evidence of increased susceptibility to IDs in persons with NCDs (Remais *et al.*, 2013). Even though certain risk factors of NCDs such as obesity are indicative of wealth status, studies from India have long highlighted that risk factors for NCDs are also present among those of the lower socio-economic status (Reddy, 2002). For instance, in Accra, the capital city of Ghana, a gradient of ID and NCD convergence has been recorded. High disease burden in relation to chronic disease has been reported among individuals from the high socio-economic class, populations from the lower socio-economic class tended to experience both high infections and NCD burdens, the latter mainly resulting from hypertension and obesity (Agyei-Mensah & De-Graft Aikins, 2010; de-Graft Aikins, 2007).

The epidemiologist Philip Sartwell, asserted that the distinction given to separate acute from chronic or infectious diseases from non-communicable diseases remain arbitrary and that the epidemiological methods useful for ID and NCD differ superficially (Remais *et al.*, 2013). Continuous surveillance of NCDs and IDs co-morbidity in LMICs would generate the empirical data required to better comprehend the dual disease burden and to target coordinated care. In instances where IDs and NCDs

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are endemic, focussing on vulnerable populations by strengthening social protections and improving access to health services is very critical (Remais *et al.*, 2013)

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2.6 Pathogenesis of *Schistosoma haematobium* parasite

Schistosoma haematobium, the causative agent of urinary schistosomiasis is the most important helminths causing the highest mortality worldwide (Nyarko *et al.*, 2018; Dent & King, 2007). *S. haematobium infection* is responsible for over 60% of all schistosomal diseases because many endemic communities have favourable environmental conditions, the intermediate host and high-risk populations. It has been estimated that 150,000 annual deaths are due to urinary schistosomiasis renal failure (Ray *et al.*, 2012; Dayan, 2003). Urinary schistosomiasis affects communities whose occupation is mostly irrigated crop farming, fish farming implying that the frequency of contact with contaminated water is high (Boelee & Madsen, 2006).

Schistosoma haematobium ova contains catechol oestrogen. Since this hormone is foreign (xeno-estrogen) to human with urinary schistosomiasis, the liver produces catechol-o-methyl transferase to degrade the catechol oestrogen. In the process, two intermediates (quinone) compound is formed. One of the intermediates is stable and the other unstable. The unstable intermediate with a genotoxic property is an electrophile. The DNA of the urothelial cells donates electrons to the quinone through a very stable bond in the major groove of DNA of urothelial cells. The DNA is damaged in the process leading to mutation in cell cycle genes (proto-oncogenes). Mutation of proto-oncogenes results in the formation of oncogenes. Unlike proto-oncogenes which confines proliferation in the basal urothelium only, oncogenes permit proliferation in the entire urothelium leading to the formation of tumour. Oncogenic activation in urothelial cells leads to malignant transformation resulting in one of several of urothelial neoplasm

Urinary schistosomiasis has chronic evolution with variable severity. Although preventable and treatable, in chronic infections, urinary schistosomiasis has series of pathogenesis. The cercariae

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penetrate the unbroken skin by secreting serine protease that digest elastin in dermis eliciting cutaneous inflammation known as cercariae dermatitis. This is characterised by reddening and itching of the skin in contaminated water. Infertility in genital schistosomiasis with increased risk of HIV infection due to compromised genital mucous membrane, kidney blockage and organ deformities resulting in organ dysfunctions (Badawy *et al.*, 2019; Ahmed *et al.*, 2014;; King , 2010; Salter *et al.*, 2000)**.**

Invasion of the venous plexus of the urinary bladder by adult worm result in iron deficiency anaemia due to loss of whole blood through haematuria and most importantly, malnutrition and failure to achieve genetic potential for growth in children. Most importantly deposition of *S. haematobium* ova in urinary bladder wall results in debilitating and irreversible clinical manifestations. Bladder pathology initiation is made possible by CE metabolism with ulceration in bladder, followed by chronic inflammation and subsequent healing by fibrosis manifesting as hyperkeratosis and squamous metaplasia due to tissue adaptation. The microenvironment change further results in mutation in tumour suppressor and proto-oncogenes as a result of DNA damage (Badawy *et al.*, 2019; Ahmed *et al.*, 2014; King C., 2010). Mutation in these genes results in over or under elaboration of the protein they encode.

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2.6.1 Conceptual framework for bladder pathology in urinary schistosomiasis

S. haematobium ova contains catechol oestrogen. Since this hormone is foreign (xeno-oestrogen) to human with urinary schistosomiasis, the liver produces catechol-o-methyl transferase to degrade the catechol oestrogen. In the process, two intermediates (quinone) compound is formed. One of the intermediates formed is stable and the other unstable. The unstable intermediate with a genotoxic property is an electrophile (electron loving). The DNA of the urothelial cells donates electrons to the quinone through a very stable bond (CpG: cysteine -phosphodiester-guanine) in the major groove of DNA of urothelial cells of urinary bladder. The DNA is damaged in the process that resist repair. This leads to mutation in cell cycle genes (proto-oncogenes) that control cell division. Mutation of proto-oncogenes results in the formation of oncogenes. Unlike proto-oncogenes which confines proliferation in the basal urothelium only, oncogenes permits proliferation in the entire urothelium leading to the formation of tumour. Oncogenic activation in urothelial cells leads to malignant transformation resulting in one of several of bladder pathologies.

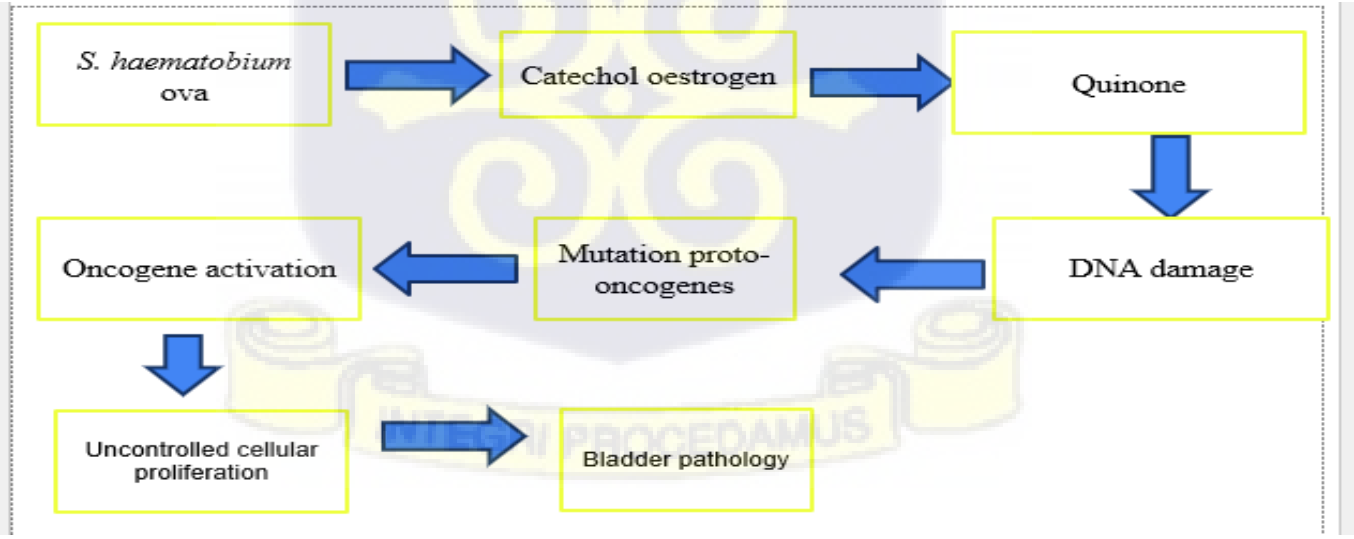

Figure 3: Conceptional framework flow chat

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2.7 The anatomy and histology of urinary bladder

The urinary bladder is a muscular sac in the pelvis just above and behind the pubic bone anatomically. Superior to the muscular layer of urinary bladder is the connective tissue and epithelium scaffold. Urine formed in the kidney passes through a pair of ureters (left and right) that connects kidney to the urinary bladder where urine is stored till voided. Voided urine leaves the urinary bladder through the urethra. The lumen of the ureters and urethra are also lined by transitional/urothelial cells which changes shape depending on whether the urinary bladder is empty or not (Lukacz *et al.*, 2011). The urinary bladder wall is made up of transitional epithelium, lamina propria and submucosa histologically (**Figure 3**). When the subepithelial structures (basement membrane, connective tissue, muscles) are stretched especially when the bladder is full, the urothelium is flattened giving the bladder its barrier property which makes it possible to store urine till voided (Banga J., 1998).

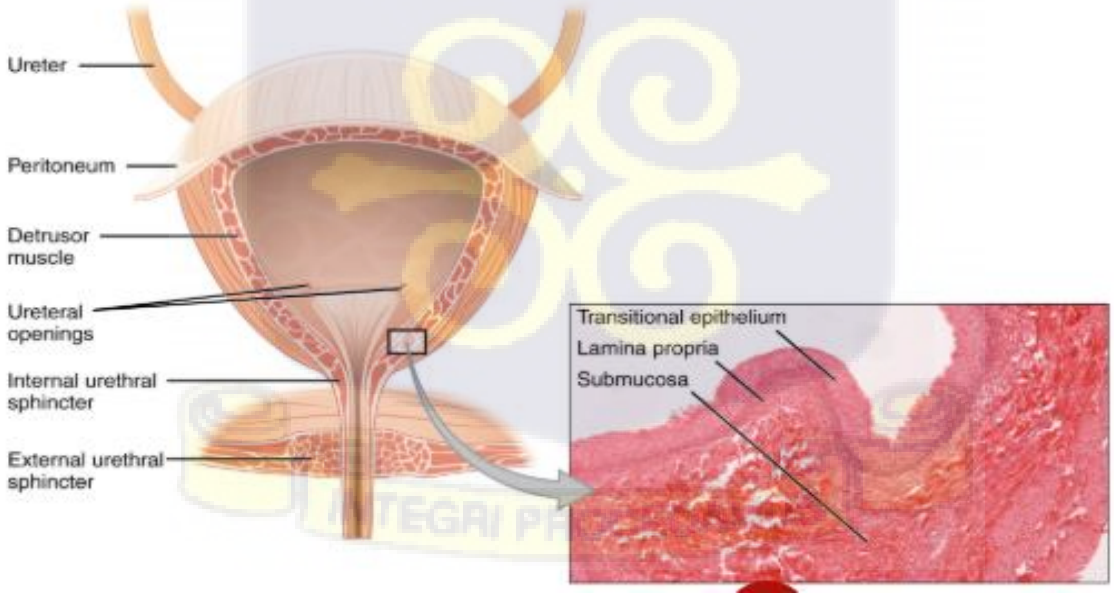

Figure 4: The gross anatomy and histology of urinary bladder. Gross anatomy and histology

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Source: Adapted and modified https://opentexbc.ca/anatomyandphysiology/chapter/25-2-gross-anatomy-of-urine-trnsport (date retrieved: 22/11/2020).

The transitional epithelium of the urinary bladder is made of multi-layered urothelial cells which is impermeable whether the normal bladder is full and empty. But distensible and mature at different levels; basal, intermediate and superficial urothelial cells (Winder, 2014). The apical layer contains urothelial cells also known as umbrella cells. The impermeable nature of the intermediate layer of the urothelium confers a barrier property protecting the underlying structures beneath the urothelium from toxins and microorganisms in stored urine (Hodges *et al.*, 2010). This barrier property like any other epithelial cells is made possible by specialized junctions on lateral surfaces of the urothelial cells in the form of junctional complexes (Zonula occludens/ tight junctions, Zonula adherens/ intermediate junction, Macula adherens/ desmosomes), gap junctions and lateral interdigitations. These junctions ensure regulated movement of ions and metabolites but prevent toxins and microorganisms across the urothelium. These junctions in disease state like in bladder pathologies, loose the property that confers barrier to the urothelium therefore immature urothelial cells are shared easily in urine. The urothelial cells have central nuclei with scanty cytoplasm evenly distributed around nuclei. The normal urothelium is polarized.

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2.8 Bladder pathology: A progressive disease in urinary schistosomiasis

Bladder pathologies due to urinary schistosomiasis can be epithelial and subepithelial in origin. Epidemiological, experimental, histopathological and cytological evidences support these correlation between urinary schistosomiasis and bladder pathologies (Botelho *et al.*, 2018; Kyritsi *et al.*, 2018; Gouveia *et al.*, 2015; Botelho *et al.*, 2010; Sherif, 1975). Therefore, diagnosis of bladder pathologies can be done cytologically or histologically. Histological diagnosis can reveal both epithelial and subepithelial bladder pathologies associated with urinary schistosomiasis because urinary bladder wall biopsy is used. Cytological diagnosis of bladder pathologies in urinary schistosomiasis (Tetteh-Quarcoo *et al.*, 2019; Sullivan *et al.*, 2010) is however limited because epithelial cells exfoliated in urine are used hence the presence of bladder pathologies in subepithelial tissue is missed but has an added advantage of been non-invasive (Sullivan *et al.*, 2010; Tzanetou *et al.*, 2007).

Epithelial bladder pathologies are progressive diseases and occur in a multistep process from precancer lesions and progressing to cancer lesions. The epithelial precancer lesions in urinary schistosomiasis are squamous metaplasia (SM), hyperkeratosis and UD in order of severity. Persistence, recurrent infection and lack of treatment of urinary schistosomiasis also contributes to progression of precancer bladder pathologies to bladder cancers (Elsebai I., 1977). The cancer lesions in bladder pathologies due to urinary schistosomiasis are SCC or TCC/UCC in rare cases (Kovalchuk O., 2016).

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2.9 Urinary schistosomiasis and associated bladder pathologies in Africa

Over 80% of the global infection with *S. haematobium* aetiologic agent for urinary schistosomiasis as an acute disease with subsequent development of bladder pathologies in chronic infections is in Sub-Saharan Africa. Although limited data exits, the prevalence of schistosomal bladder tumours are implicated in many parts of Africa where *S. haematobium* infection is endemic. In *S. haematobium* free settings, bladder cancers are prevalent after 6 decades of one's life. In areas where *S. haematobium* is hyper endemic however, the occurrences of bladder cancers happen a few years after 4th decade of life (Botelho *et al.*, 2018). However, some epidemiological and experimental studies have found strong association between urinary schistosomiasis and bladder pathologies. In Egypt, a study conducted revealed urinary schistosomiasis as a risk factor to developing SCC in most cases than UCC (1st mentioned in introduction)(OR ¼, 1.8; 95% CI, 1.2-2.6) (Zheng *et al.*, 2012). The mechanism underlying *S. haematobium* infection related tumorigenesis must be understood so that it serve as a tool for developing new vaccines or new drugs to combat the disease (Boelee *et al.*, 2006).

In Nigeria, a study found an association between urinary schistosomiasis and bladder pathologies with the frequency of malignancies been higher in people with light intensity of *S. haematobium* infection although not statistically significant (Onile *et al.*, 2016). In Tanzania, a correlation of almost 45% was found between urinary schistosomiasis and SCC, the difference was statistically significant (Rambau *et al.*, 2013). In 2016 an ultrasound detection of bladder pathology among 257 people with urinary schistosomiasis, prevalence rate of bladder pathology was higher in females than males although not statistically significant (Onile *et al.*, 2016). A study in Egypt revealed urinary schistosomiasis and other risk factors (smoking cigarette and water pipe) as a basis for development

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squamous cell carcinoma and transitional cell carcinoma respectively, there was significant difference urinary schistosomiasis and SCC ((Zheng *et al.*, 2012).

In a study in Kenya, there was no significant sex difference in prevalence of hyperkeratosis or atypia but there was significant difference between metaplasia in the elderly and urinary schistosomiasis (Hodder *et al.*, 2000). A retrospective histological study in Tanzania there was statistical difference between SCC and urinary schistosomiasis (Rambau *et al.*, 2013). In Zambia, a 5 years study revealed 75% differentially diagnosed SCC, 2/3 of these had schistosomal ova in bladder biopsies (Bhagwandeen S., 1976). An experimental study establish causal relationship between urinary schistosomiasis and development of bladder cancer, the difference which was statistically significant (Hicks *et al.*,1980).

Review of literatures revealed a sequelae of bladder pathologies manifesting squamous metaplasia and urothelial dysplasia among others in urinary schistosomiasis (Brand, 1979). In an experiment, mouse bladder wall injection with *S. haematobium* eggs led to urothelial hyperplasia, a potentially pre-cancerous lesion (Fu *et al.*, 2012). In a review study, keratinizing squamous metaplasia which has been implicated as prerequisite in developing SCC in urinary schistosomiasis lacks evidence but the presence of UD had concrete evidence in the development of SCC in *S. haematobium* infection (Ahmad *et al.*, 2008).

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2.10 Urinary schistosomiasis and associated pathologies in Ghana

Not much studies have been done in Ghana to establish association between urinary schistosomiasis and bladder pathologies. Tetteh-quarcoo *et al.* (2019) in a very recent study stated that children suffering from urinary schistosomiasis also have hyperkeratosis and squamous metaplasia, which is a prerequisite for developing bladder cancer. In a pilot study by Zhong *et al.* (2013), it was found that RASSF1A and TIMP3 found at the promoter region of tumour suppressor gene was methylated, making it less efficient in playing its role of suppressing tumour growth. Therefore, there is uncontrolled cellular proliferation in urinary schistosomiasis. A study in Ghana a decade ago has revealed children within the age group of 6-17 being the most vulnerable to *S. haematobium* infection (Nkegbe E., 2010).

In another study, radiological imaging of the bladder of over 1200 people suffering from urinary schistosomiasis revealed some bladder pathologies which were treated using chemotherapy. Some of the pathologies were resolved after treatment however over 60% of those had upper urinary tract pathology did not resolve and needed retreatment (Wagatsuma *et al.*, 1999). In a five (5) case studies of minors with urinary schistosomiasis in Ashanti Region, Ghana presented to the hospital with end stage renal failure and bladder pathologies. This study recommended mass bladder screening among other organs of especially children *(*Antwi *et al.*. 2014).

A retrospective histopathological review of surgical schistosoma-associated tissues, urinary schistosomiasis constituted over 36% of all cases. Cumulatively this study found 20% of all histological tissues that contained *Schistosoma* ova associated with cancers. Of the *Schistosoma*-associated cancers cases, SCC was over 80% but the study failed to apportion the rate of cancers due to urinary schistosomiasis (Der *et al.*, 2015). Another studyhypothesized cytological abnormality

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among children could lead to bladder cancer in the future (Tetteh-Quarcoo *et al.*, 2019). What then is responsible for the initiation of carcinogenesis of bladder cancer in urinary schistosomiasis? All these studies in Ghana did not identify the substance in *S. haematobium* that might be responsible for the pathogenesis. Oestrogen in general has been implicated in a lot of cancers and catechol oestrogen found in *Schistosoma* egg antigen (SEA) has been hypothesized to be an initiator of bladder cancer (Travis & Key, 2003).

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2.11 Children vulnerability to urinary schistosomiasis

Children especially males of school going age are most vulnerable to *S. haematobium* infection due to frequent contact with contaminated water through daily activities (Yirenya-Tawiah *et al.*, 2011; Nkegbe E, 2010; Hodder *et al.*, 2000). The skin is covered by stratified squamous epithelium matures under influence of oestradiol but there are little oestradiol levels in children so squamous cell differentiation may not be terminal. Claudin and other junctional complex proteins of the epithelium are produced by terminally differentiated epithelial cells confer the barrier property to the skin. This implies that the barrier property of skin of children is weak (Braniste *et al.*, 2009). Epithelial junctional complexes serve as target for pathogen manipulation to penetrate the skin and they survive as they proliferate, because the skin is permeable (Furuse *et al.*, 2002). One such protein is claudin that form the main component of junctional complex protein is digested by cercarial serine protease and elastase (Salter *et al.*, 2000).

The urinary schistosomiasis there is decrease transcription of uroplakin genes and as well as decrease transcription of tight junction genes claudin and junctional adhesion molecules (Ray *et al.*, 2012). Another taught suggest that human mannose binding lectin (MBL) protein which is involved in *S. haematobium* recognition as first line of defence is low in children with infections. Low MBL together with MBL-associated serine protease 2 (MASP-2) complement lectin activation cascade is not effective. Therefore it stands to reason that there will be reduced activity of the complement against *S. haematobium*, resulting in chronic urinary schistosomiasis (Uchendu *et al.*, 2017). Malnutrition as well as anaemia are symptoms of urinary schistosomiasis in children and this affects the cognitive function, making it more likely for them to drop out of school.

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2.12.1 Bladder pathology, urinary schistosomiasis and gender

The prevalence of bladder carcinoma is said to be high in males than in females. The differences in incidence of bladder malignancies between males and females can be attributed to exposure to smoke from tobacco and industrial carcinogens but an Egyptian study that used gender as variable with smoking found no significant difference between males and females in developing SCC (Kyritsi *et al.*, 2018). In a study in United State of America, an experiment was performed to see if *S. haematobium* egg induce bladder urothelial abnormalities was dependent on host gender. Gender (being female) influenced p53 activity in *urinary* schistosomiasis (Honeycutt *et al.*, 2015).

Bladder pathologies is made up of epithelial and subepithelial tumours. The epithelial bladder pathologies are hyperkeratosis, squamous metaplasia, urothelial dysplasia, transitional cells/urothelial cell, carcinoma, squamous cell carcinoma. The subepithelial bladder pathologies are chronic cystitis, cystitis glandularis, von Brun nest or bud, polypoid cystitis among others. In cytology, among the epithelial lesions associated with urinary schistosomiasis are keratinizing squamous metaplasia (K-SM), non-keratinizing squamous metaplasia (NK-SM), hyperkeratosis, squamous cell carcinoma (SCC), urothelial dysplasia (UD) and occasional urothelial cell carcinoma (UCC) which is burdened with inconsistencies in grading worldwide. An attempt has been made by the Paris System for Reporting Urinary Cytology of urothelial cells and The Bethesda System of Reporting Urine Cytology to standardize the reporting of urine cytology but to no avail (Department of Pathology, UGMS).

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2.12.2 Squamous metaplasia in urinary schistosomiasis

Under physiologic conditions, the bladder epithelium is lined by urothelial cell. The basal urothelial cells have multipotent ability so can proliferate forming another type of epithelial cells if the microenvironment changes (Weerakoon *et al.*, 2015; Santos & Fernandes, *et al.*, 2014). In urinary schistosomiasis, the presence of CE metabolite, quinone, an abnormal extracellular stimulus, changes the microenvironment of the bladder causing chronic irritation (Lemon *et al.*, 1992). The basal urothelial cells layer identifies change in microenvironment and react to adapt to the change in environment. The basal urothelial cells adapt to the change in microenvironment proliferates replacing urothelial cells with stratified squamous metaplastic cells a phenomenon known as squamous metaplasia (Kawanishi *et al.*, 2017; Sui *et al.*, 2017). Metaplasia is the replacement of normal mature cell with another type of mature cell type. Squamous metaplastic cells can be keratinizing or non-keratinizing. Squamous metaplasia is physiologic when the cytoplasm is non-keratinizing or pathologic when it is keratinizing. Keratinizing squamous metaplasia has the potential to develop into urothelial dysplasia which can also progress to bladder cancer SCC (Arora *et al.*, 2019; Ahmad *et al.*, 2008; Hodder *et al.*, 2000). Studies in USA and Ireland support the association between urinary schistosomiasis and squamous metaplasia (Honeycutt *et al.*, 2015; Khan *et al.*, 2002).

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2.12.3 Hyperkeratosis in urinary schistosomiasis

Hyperkeratosis is induced by chronic inflammation in urinary schistosomiasis and lack of effector cell elicit receptor immune response and can manifest cytologically as granulomatous inflammation and hyperkeratosis (Rambau *et al.*, 2013). Granulomatous inflammation may heal by fibrosis that cause stasis of urine resulting in secondary bacterial infection in urinary schistosomiasis or plays varying roles in initiation, promotion, invasion and metastasis in bladder carcinogenesis especially SCC in urinary tract infections including urinary schistosomiasis (Zhu *et al.*, 2012). Hyperkeratotic cells are abnormally keratinized and without nuclei microscopically. During chronic *S. haematobium* infection, the trigone undergoes squamous metaplasia resulting in the change of urothelial cells to non-keratinizing metaplastic squamous cells (N-KSC) with loose interdigitations among cells. Chemicals, allergens, hormones, toxins, potassium ions in urine seep through the bladder tissues because it has lost its waterproof properties and bind to receptors of N-KSC and in the process keratinize them. According to Ahmad *et al.* (2008), hyperkeratosis is a premalignant condition which can develop into SCC if not treated. Hyperkeratosis resist desquamation and can progress with time into SCC (Wechsel *et al.*, 2000). DNA damage of urothelial cells triggered by semiquinone induces chronic inflammatory response that produces excess reactive oxygen species (ROS) (nitric oxide, superoxide, hydrogen peroxide) because the extent of its production exceeds its neutralization by antioxidants. Excess ROS form free radicals that further damages urothelial cell organelles including the DNA that can initiate carcinogenesis (Kawanishi *et al.*, 2017).

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2.12.4 Urothelial dysplasia in urinary schistosomiasis

Urothelial dysplasia is a phrase that describes a morphology of urothelial cell without taking into consideration the biology or the biochemical reaction of the cell. The Greek word '*Dys'* means bad and '*plasia* 'means molding. The term had generated a lot of discussions and discord leading to the agreement that dysplasia is classified as severe, if the trigger is a carcinogen. Since *S. haematobium* has been classified as carcinogen, urothelial dysplasia associated with urinary schistosomiasis is a precancer lesion. A study in Portugal found an association between urinary schistosomiasis and urothelial dysplasia (Botello *et al.,* 2013; Botelho *et al.,* 2011). Urothelial dysplasia was observed in a study in Seoul, Korea where the bladder wall of mice was injected with *S. haematobium ova* (Chala *et al.,* 2017).

2.12.5 Intensity of S. haematobium infection and severity of bladder pathologies

Urinary schistosomiasis and bladder pathology my share common symptoms like haematuria, dysuria and pain accompanying micturition (Onile *et al*., 2016). Similarity in symptom will prevent early diagnosis of bladder pathologies. Intensity of infection can dictate disease progression. Few worms lay few eggs, many worms lay many eggs hence high intensity of infection. Intensity of infection influence progression of the bladder pathology. Some experimental observation made showed that the extent of DNA damage is correlated to intensity of infection.

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2.12.6 Bladder cancer associated with urinary schistosomiasis

The World Health Organization-International Agency for Research on Cancer (IARC) confirmed that chronic infection with *S. haematobium* causes cancer of the urinary bladder (WHO. 1994). This is an NTD-associated cancer, a neglected cancer as well as and a major public health problem of Africa's most impoverished countries (Botelho & Sousa, 2014). Bladder cancer ranks third among all cancer deaths. Bladder cancer exists as either TCC/UCC or squamous cell carcinoma (Botelho *et al.*, 2017). In communities where *S. haematobium* is endemic, SCC is most prevalent as compared to TCC/UCC in non-endemic communities (Kyritsi *et al.*, 2018; Vale *et al.*, 2017). A study in London disputed the association between urinary schistosomiasis and SCC, rather it found an association between urinary schistosomiasis and fibrotic change (Brand, 1979). In a study in Egypt, there was significant decrease in SCC when *S. haematobium* transmission was controlled suggesting a strong association between urinary schistosomiasis and SCC (Jalloh *et al.*, 2020).

In the year 2012, a study in Egypt among over 2,000 confirmed bladder cancers at 95% confidence limit, the adjusted odd ratio was 2.12 (AOR=2.12) (Kyritsi *et al.*, 2018). In a similar case control study in Egypt, SCC associated with urinary schistosomiasis at confidence interval of 1.2-3.0, it was statistically significant to develop SCC associated with urinary schistosomiasis when one was a female (OR: 1.9) and men (OR: 1.4) (Zheng *et al.*, 2012; Botelho *et al.*, 2009). In a similar study in Sudan, among 155 bladder cancers associated with urinary schistosomiasis, 87 was SCC with a statistically significant risk of developing SCC when one had urinary schistosomiasis ($p<0.000$) (Hassan, *et al,* 2013).

It has been reported that, depending on the stage of tumour, the survival rate for SCC is between 32-65% (Lanz *et al.*, 2019). Squamous cell carcinoma can metastasize to kidney if not treated resulting

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in kidney failure, dialysis and eventually death (Bournakis *et al.,* 2011). This is in support of a study in Tanzania (Rambau *et al.,* 2013). This finding of SCC is similar to studies from Egypt which the prevalence of SCC was about 36% (Kyritsi *et al.,* 2018). A 65% prevalence rate of SCC was observed in people with urinary schistosomiasis in an endemic community in Nigerian (Mungadi and Malami, 2008).

2.12.7 Hormonal carcinogenesis

Oestrogen exists as endogenous or exogenous hormone. They are both metabolized by cytochrome P450 enzymes but unlike endogenous, the metabolism of exogenous is irreversible reaction. The origin of exogenous oestrogens is either plant based, industrial chemicals as well as infectious agents among other substances. The mechanism of hormonal carcinogenesis is different from other substances. In hormonal carcinogenesis, no specific initiator is required. There is high rate of DNA error during cell proliferation that resist repair with subsequent mutation and malignant phenotyping. MALIGNNT phenotype results in somatic mutation. Hormonal stimulation of cell proliferation is progressive (Henderson & Felguson (2000).

2.12.8 General pathogenic mechanism of bladder cancers

Oestrogen has been implicated in most epithelial cancers (Cavalieri & Rogan, 2016). Schistosoma Egg Antigen (SEA) contains CE that is hypothesized to be responsible for bladder pathologies in urinary schistosomiasis (Botelho *et al.,* 2009). Since CE is a foreign compound, it is metabolized by carcinogen-enzyme COMT from the liver to form compound 4-hydroxylestradiol and 2-

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hydroxylestradiol The 4-hydroxylestradiol is an unstable intermediate which is further oxidized to form 3,4-quinone, the major carcinogenic metabolites of oestrogen with genotoxic property (Santos *et al.*, 2014). 3,4-quinone is electrophilic which have high affinity for DNA so it binds to form depurinating oestrogen-DNA adduct in the bladder damaging the DNA in the process (Penning, 2017; Cavalieri & Rogan, 2016; Gouveia e*t al.*, 2015; Dawling *et al.*, 2001). The DNA damage resists deletion by apoptosis as well as resist DNA repair because the bond formed between DNA and quinone is in the major groove. There is further mutation in tumour suppressor gene and protoncogenes (Kawanishi *et al.*, 2017; Botello *et al.*, 2011). Point mutation of protoncogenes at codons 12, 13, 61, 113-117 activates oncogenes (Lodish *et al.*, 2000). Oncogene is locked in the 'on' signalling conformation. This results in abnormal cellular proliferation of bladder epithelium. Epithelial cells proliferation is controlled by complex intracellular as well as extracellular signalling network whose disruption is attributed to malignant transformation (Sun *et al.*, 2019; Botelho *et al.*, 2009). The oestrogen -DNA adduct formed as a result of presence of CE in bladder activate apurine sites (purinergic receptor) making the oestrogen -DNA adduct mutagenic (Cavalieri & Rogan, 2016).

If DNA is damaged as occurs in the case of urinary schistosomiasis, extracellular nucleotide (ATP) binds to purinergic receptors that couples with G- protein. P2X ion channels are epithelial that regulate salt and water transport in and out of the cell. Formation of adduct in urothelial cells in effect opens P2X ion channels non-selectively to Calcium ions resulting in increased cytosolic calcium ions leading to increase intracellular ATP release to stimulate metabolic activities of the urothelial cells without inhibition (Antonio & Egan, 2016; Winder *et al.*, 2014). Extracellular ATP also activates the receptors for epidermal growth factors that signals urothelial cells to elicit sustained proliferation (immortalization) through growth factor induced phosphorylation pathways (Teng *et al.*, 2015; Mohammadi *et al.*, 1992). There is also reduced apoptosis and silencing of

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tumour suppressor genes which is characteristic of carcinogenesis in urinary schistosomiasis induced bladder pathology (Feitelson *et al.,* 2016). The DNA -adduct can leave the urothelial cells and dissolve in urine so it is measurable spectrophotometrically (Cavalieri & Rogan, 2006).

The development of bladder cancer in urinary schistosomiasis is a complex event in a multistep process. Hyperkeratosis, squamous metaplasia; urothelial dysplasia and bladder cancer are induced by presence of semiquinone, CE metabolites that changes the microenvironment of bladder (Park, 2018; Kovalchuk,1 2016; L8iehr, 1990).

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2.13 Biomarkers

The use of biomarkers in basic and clinical research together with clinical practice has become very common. This is because, the use of biomarkers as primary endpoints in most clinical settings has currently been accepted (Strimbu & Tavel, 2011). Biomarker is a term consisting of a combination of words meaning 'biological marker', referring to a "broad subcategory of medical signs – that is, objective indications of medical state observed from outside the patient – which can be measured accurately and reproducibly (Strimbu & Tavel, 2011). In 1998, the National Institutes of Health Biomarkers Definitions Working Group defined a biomarker as "a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention (Strimbu & Tavel, 2011).

In their report on the validity of biomarkers in environment risk assessment, the WHO has stated that a true definition of biomarkers includes "almost any measurement reflecting an interaction between a biological system and a potential hazard, which may be chemical, physical, or biological. The measured response may be functional and physiological, biochemical at the cellular level, or a molecular interaction (World Health Organisation (WHO) International Programme on Chemical Safety (IPCS), 1993).

2.13.1 Prognostic and diagnostic biomarkers in the surveillance of bladder cancers

Bladder pathologies are high in communities where risk factors are high, in environments where there is exposure to carcinogenic substance like nicotine in cigarettes smoke, industrial waste such as nitrosamine and infectious agents such as *S. haematobium* among others. Whilst bladder

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pathologies induced by smoking and industrial waste exposure originate from epithelium to subepithelial tissues, bladder pathologies due to urinary schistosomiasis originates from subepithelial tissues to epithelial tissues in urinary schistosomiasis. Therefore incidence of non-epithelial bladder pathologies is high in *S. haematobium* endemic communities (Miyazaki & Nishiyama, 2017). Urine cytology has high diagnostic accuracy in high grade epithelial bladder pathologies with an average sensitivity of about 85% and over 90% specificity (Sullivan *et al.*, 2010; Planz *et al.*, 2005). In low grade lesions however, the sensitivity can be as low as 4%. Some inflammatory and benign conditions with features that mimic bladder cancer may result in false positive. Cystoscopy is another effective tool for diagnosing bladder pathologies at all levels, but the limitation to this is that, it involves invasive procedure (Cicione *et al.*, 2013). These limitations does not make cytology appropriate diagnostic tool for screening for potential bladder cancer risk (Lee & Kim, 2020). To overcome these limitations, there is the need to research into biomarkers to be used as prognostic marker in population based screening for bladder pathologies in especially *S. haematobium* endemic areas and for precision diagnosis in low grade bladder pathologies (Lotan *et al.*, 2017).

The source of materials to use to assess biomarker for bladder pathologies is either the bladder cells, protein, gene or nucleic acid (de Oliveira *et al.*, 2020). The ideal biomarker must meet the criteria of been cost effect, fast to process and easy to interpret and sample obtained through non-invasive means (Califf R., 2018). Fluid biopsies is recommended for demonstration of biomarkers of bladder pathologies in modern medicine (Saarenheimo *et al.*, 2019; Di Meo *et al.*, 2017). The interaction between urine and bladder tumour cells makes it suitable as biomarker for screening purposes. There is growing interest in liquid biopsies concept due to its importance in disease progression and follow ups. Bladder pathologies progression from benign to malignant state can be assessed using liquid assays from exfoliated cells, protein, genes, metabolites and nucleic acids. Extracellular vesicles has

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also been identified as source of biomarkers in bladder pathologies (de Oliveira *et al.*, 2020). There are some Food and Drug Board (FDB) approved and commercially available biomarkers as well as non-FDA approved biomarkers under research. Urovysion is a brand that demonstrate aneuploidy of chromosome 3, 7, 13 and 9p21 by fluorescent in situ hybridization (FISH). ImmunoCyt/uCyt+ test detection of carcinoembryonic antigen (CEA) and mucins by immunohistochemistry (IHC), bladder tumour antigen (BTA).

2.13.2 Candidate biomarkers for schistosomiasis associated bladder pathologies

A study by Santos *et al.* (2014) identified urinary oestrogen metabolites of *S. haematobium* worms that down-regulate oestrogen receptors (ER) **α** and **β** in oestrogen responsive cells in vitro by mass spectrographic analysis (Santos *et al.*, 2014). Similarly, molecules looking like oestrogen have been found in the blood and urine samples of people infected with schistosomiasis. These molecules were found to be able to trigger cancer and was also correlated with infertility in women (Botelho *et al.*, 2015; Santos *et al.*, 2014). Approximately eighty-two percent (82%) of individuals having bladder carcinoma were found to harbour *S. haematobium* eggs within the wall of the bladder (Eissa *et al.*, 2015). The existence of eggs was related to the development of cancer at a younger age and a predominance of squamous cell carcinoma, relative to egg-negative cases. A higher degree of adenocarcinoma has also found in schistosomal bladder carcinoma (Ahmad *et al.*, 2008).

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2.13.3 Proto-oncogenes

According to American Cancer Society, 2008, one of every two men and one among every 3 women is likely to develop cancer in lifetime. Over 69 cancer genes are genes mutated from germ line origin and over 340 gene associated with somatic mutation. However, mutation in 2 of such genes play key roles, proto-oncogenes and tumour suppressor gene. Proto-oncogene is a healthy gene made up of sequence of DNA necessary for proper cell growth and cell division among others. Proto-oncogenes encode a protein that control proliferation in normal cells). In embryogenesis, because of high rate of cell proliferation and growth, the expression of proto-oncogenes is very high. Proto oncogenes under normal circumstances are switched off after its activity is completed. There are over 40 types of proto-oncogenes but mutation in a few of them implicated in cancers are RAS, Myc, Her2 and cyclin D1 proto-oncogenes. All mammalian cells express 3 types of RAS proto-oncogenes known as H-RAS, K-RAS and N-RAS (Saeed *et al.*, 2019; Anderson *et al.*, 1992).

2.13.4 Mutation of proto-oncogenes

Exposure to some environmental triggers such as steroids (oestrogen and progesterone), harmful radiation, dyes, fuel exhaust, asbestos, oncogenic viruses can result in mutation of proto-oncogenes but not all mutations may result in cancers. Mutation of genes that codes for protein responsible for forming intermediates in carcinogenesis is an advantage to the host. Proto oncogenes after performing its function of call proliferation in cell cycle is switched off indefinitely under physiologic conditions. During mutation of the gene after exposure to the triggers mentioned above, reactivation

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of proto-oncogene after switching off can result in cancers. If protooncogenes are mutated, they evolve to oncogenes which causes excessive cell proliferation (Chail H. 2018).

2.13.5. Abnormal cellular proliferation and expression of Ki67

Ki67 is a non-histone nuclear protein or antigen responsible for cellular proliferation or growth fraction is confined to the basal epithelium/reserve cell under physiologic condition (Sun *et al.*, 2019). Ki67 is encoded by the gene MKI67 in human located on the long arm of chromosome 10 (10q25) is found in all growth phases of cell cycle where there are actively dividing cells with the exception of quiescent phase (G0) (Fonatsch , Duchrow , Rieder & Schlüter, 1991). Beginning in the mid G1 of cell cycle, the level of Ki67 increases through S phase getting to the peak in M phase. In proliferating cells, Ki67 protein is associated with ribosomal ribonucleic acid (RNA) transcription (Bullwinkel *et al.*, 2006).

Location of Ki67 protein varies depending on phase of cell cycle. Suring interphase, Ki67 can be demonstrated within nucleus whereas in whilst in M phase the protein is located on the surface of chromosomes (Larsen *et al.*, 2001). Sequencing of complementary deoxyribonucleic acid (cDNA) of Ki67 protein, 2 isoforms of messenger ribonucleic acid (mRNA) with open reading frames of 9,768 and 8,688 base pairs (bp) encoding for this proliferating protein with molecular weights of 365,761 Dalton (D) and 319,508 D respectively (Schlüter *et al.*, 1993). The most outstanding feature of Ki67 antigen is 'the Ki67 repeats' contained in the large exon 13 that made up of more than 70% of both isoforms. About 43-62% of amino acids of 'the Ki67 repeats' are identical. Within these

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repeats is a sequence of 22 amino acids called the 'Ki67 motif, a 66 base pairs (bp) motif. This motif is highly preserved among species, and it is the target for Ki67 antibodies.

Under disease condition the barrier property of superficial and intermediate urothelium is lost and assumes a proliferative role so Ki67 can be demonstrated in all urothelial cells which under physiologic conditions was confined to basal urothelium. Experiment done in Seoul, Korea confirms that *S. haematobium* induces abnormally high mitosis in urothelial cells (Chala *et al.*, 2017). In all pathologies including bladder pathologies due to urinary schistosomiasis, proto-oncogenes which controls normal cells proliferation is mutated to oncogenes leading to uncontrolled cellular proliferation because oncogene is put 'on' conformation. This is due to increase in intra- and extracellular ATP that in turn increase metabolic activities of all cell organelles resulting in hypertrophy and latter hyperplasia.

The principle of immunocytochemistry is based on antigen-antibody reaction. Some cells may contain endogenous peroxidase-like enzymes that may interfere with the final exogenous peroxidase label visualizing step, so its reaction was blocked using excess of hydrogen peroxide (substrate) at higher concentrations. The corresponding antibody raised in mouse binds with antigen (Ki67) after incubation. Excess antibodies were washed with buffer (pH 7.2). Labelled secondary antibodies to the immunoglobulin of primary antibodies' host (goat anti-mouse immunoglobulin) binds to the primary antibodies at the very site it binds to the receptor or antigen for visualization under the microscope. The label is polymerized horseradish peroxidase which provides enzyme label on secondary antibody molecule. During a secondary washing, peroxidase reacts with its hydrogen peroxide (substrate) and diaminobenzidine (DAB) which gives a dark brown insoluble precipitate at site of reaction. The precipitate is counterstained with

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Haematoxylin, dehydrated using varying grades of ethanol, cleared in xylene and mounted using DPX mountant.

2.13.6. The potential of Catechol Oestrogen (CE) as a biomarker for urinary schistosomiasis-associated bladder pathologies

The risk of developing cancer is strongly associated with xenohormones (Sahib, 2019; Mosli *et al.*, 2013). Oestrogen has been implicated in a lot of cancers (Park, 2018; Tuffour *et al.*, 2018; Ide & Miyamoto, 2015). Catechol oestrogen found in the ova of *S. haematobium* is encoded by the genes CYPA1 and CYPA2 (Cerne *et al.*, 2011). These genes have homology with the genes that encode the enzyme catechol-O-methyltransferase (COMT) belonging to the super family enzymes of cytochrome p450 synthesized in the liver. Because is encoded by 2 genes bearing homology with CE, it catalyses the breakdown of substances with catechol structure (2-hydroxylation and 4 - hydroxylation) into 2-hydroxyestridiol and 4-hydroxylestridiol semiquinone in people with urinary schistosomiasis. The semiquinone 2-hydroxyestrodiol however is a stable compound and less genotoxic (Mosli *et al.*, 2013). Further oxidation of 4-hydroxylestridiol yields an unstable semi quinone, an electrophilic that react with purine bases of DNA forming depurinating adduct with strong genotoxic properties. Formation of oestrogen adduct is an irreversible reaction that progresses to form cancers in human (Wen, 2017; Cavalieri & Rogan, 2016;; Santos *et al.*, 2014; Yuseku *et al.*,, 2001; Gori & Peters, 1975).

Since the semiquinone is an electrophile, it has affinity for a nucleophile that donates a pair of electrons to form a stable bond. Purine bases of DNA of bladder urothelial cells are nucleophilic and donate paired electrons to semiquinone, the metabolite of CE to form DNA adduct (Stachowicz-

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Kušnierz *et al.*,, 2016; Gouveia *et al.*, 2015; Botelho *et al.*, 2013). The genotoxicity of semiquinone induces mutation in cell cycle genes converting protoncogenes to oncogenes) and down regulate tumour suppressor genes (Anderson *et al.*, 1992). Hence the equilibrium that exist between programmed cell death (apoptosis) and controlled cell proliferation is lost. This results in up-regulation of abnormal proliferation (Foster J., 2000).

The presence of DNA adduct further induces chronic inflammation yielding free radicals that which has been implicated as initiator, promotor of carcinogenesis (Kawanishi *et al.*, 2017). In the process, there is the transfer of a methyl group to DNA through a bond in a region in DNA called CpG (cytosine nucleotide occurs next to guanine) island at a specific position known as fifth base within the DNA major groove (Kovalchuk O., 2016). The 'p' in CpG is a phosphodiester bond linking Cytosine in DNA to Guanine in the semiquinone to form DNA adduct. The bond is so strong that it last as long as the DNA lives. The DNA adduct reduces apoptosis and increases cellular proliferation therefore people with chronic *S. haematobium* infection are likely to develop blabber cancer if not treated (Botelho *et al.*, 2009).

There is malignant transformation of the hyperplastic urothelium in the process. CE further induces alteration in the urothelium of the bladder such that the perpendicular arrangement of urothelial cells is lost (Botello *et al.*, 2011). This results in loss of polarity of the urothelium. A study by Betolho *et al.* (2017) had shown CE metabolism yields tumour-like phenols in urothelial cells originated from parasite oestrogen–host cells interaction that induce chromosomal DNA adduction and tumour suppressor gene mutations.

Catechol oestrogen in urinary bladder is metabolized by catechol -O-methyltransferase to form 3,4-hydroxyestridiol which is further oxidized to form a semiquinone. The semiquinone is highly

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genotoxic and unstable forms DNA adduct that damages the DNA of urothelial cell with further mutation in cell cycle genes (tumour suppressor and proto-oncogenes). Apoptosis and tumour suppression is down regulated and there is up-regulation of cellular proliferation (Figure 4).

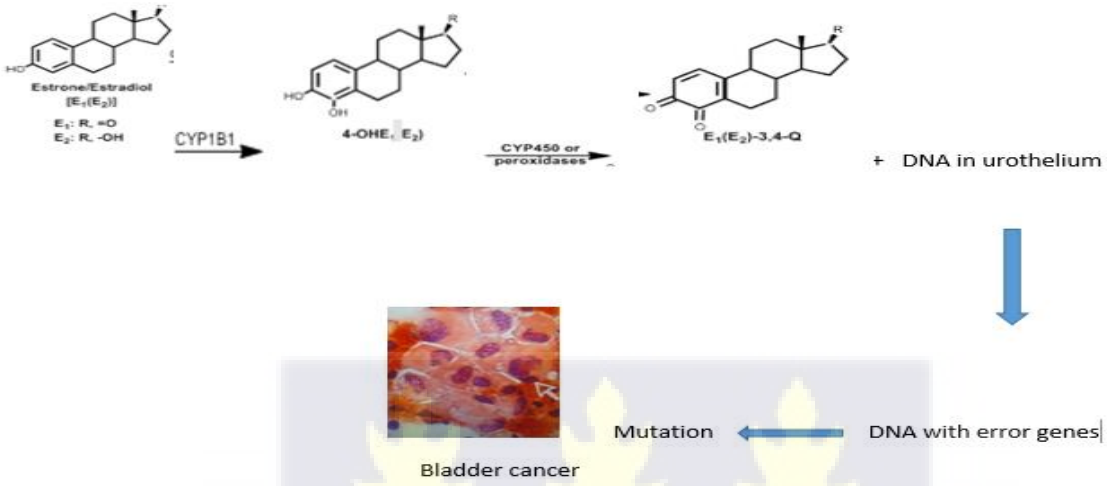

Figure 5: Mechanism of Catechol oestrogen metabolism

The first black arrow indicates conversion of CE to 3,4-hydroxyestridiol in first structure to second structure, second black arrow shows conversion of 3,4-hydroxyestridiol to semiquinone in the third structure. Thick blue arrow is conversion to DNA adduct after DNA damage. Thin blue arrow indicates mutation in DNA adduct.

Source: Adapted and modified from Botelho *et al.*, (2017) (retrieved 22/1/2020 and micrographs of thesis

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CHAPTER THREE

3.0. MATERIALS AND METHODS

3.1. Ethical clearance and consent

Two different sets of samples were used - archived bladder biopsies and community samples. The protocol (NMIMR-IRB CPN 038-19-8-19) (Appendix 1) of the study was reviewed and approved by the Institutional Review Board (IRB) of Noguchi Memorial Institute for Medical Research (NMIMR). Permission was sought from the Department of Pathology, University of Ghana Medical School, Korle-Bu Teaching Hospital (KBTH) for the usage of archived bladder biopsies. For the community samples, only adults who gave consents and children whose guardians/parents consented for their wards' participation in the study, after explaining the aims and objectives as well as benefits of the study in their language of understanding were recruited. A written child assent (Appendix 2) was obtained from children who could read and write as recommended by NMIMR-IRB as well as the guardian. Participants were well informed about the objectives and procedures of the study. They were told that participation was optional, and they were free to opt out anytime. After consenting (adult: Appendix 3, parental: Appendix 4) /assenting (Appendix 2), study participants were given a questionnaire (Appendix 5) to fill afterwards. A total number of 450 participants were recruited in the study of which 19 were dropped because they did not meet the criteria for the study.

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3.2 Study design

The study involved the use of both patients' archived bladder biopsy samples and community urine samples. Cross-sectional study was conducted on the community urine sample and retrospective study was conducted on patients' archived bladder biopsy samples.

Demographic data and bladder pathology diagnosis were retrieved from archived reports of bladder biopsies collected over 10 years. The patients' archived biopsy samples were examined using histopathology and ova count. Results of laboratory investigations were analysed using prevalence rate, Pearson chi-square, odds ratio, Spearman correlation, ANOVA and Tukey multiple comparison and ROC curve.

Demographics data on knowledge, attitude, behaviour and practices with respect to schistosomiasis transmission were collected during the community sample collection in Zenu and Weija. Urine samples and contaminated water in the endemic communities were collected and analysed. For the community urine samples, ova count, cytological as well as histopathological analyses and for HER2. Cyclin D1, and Ki67 proteins on samples positive for bladder pathologies using immunocytochemistry. Study participants diagnosed with urinary schistosomiasis were treated with praziquantel and comparison of pre and post treatment levels of CE was estimated. Results of laboratory investigations were analysed using prevalence rate, Pearson chi-square, odds ratio, Spearman correlation, ANOVA and Tukey multiple comparison and ROC curve. Data obtained through questionnaire response was analysed using descriptive statistics and chi squared.

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3.3 Study participants and collection of samples

The sources of sample for the study were two: bladder biopsies of patients who visited the Department of Pathology of Korle-Bu Teaching Hospital and urine samples of study participants who live in *S. haematobium* endemic communities/ environs of Zenu /Weija and were qualified to participate based on the criteria for the study.

3.3.1 Inclusion criteria for urine samples

Males and females aged between 6 - 65 years living at Weija and its environs in the Ga South Municipality and Zenu in Ashaiman Municipality, both being communities in Greater Accra Region, Ghana, who gave their consent as adults as well as parental consent for minors. In addition to parental consent, child accent was provided by minors who could read to give information on themselves, were all recruited into the study.

3.3.2 Exclusion criteria for urine samples

People with any history of smoking, urinary tract problems, industrial workers and people on treatment for bladder issues and those receiving treatment for urinary schistosomiasis currently and in the last 2 years were not part of the study. These persons might have been exposed to some agents that may influence the results of the research.

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3.3.3 Control participants

The people at the study site aged between 6-65 years who tested negative for *S. haematobium* infection at the study sites were used as controls.

3.3.4 Inclusion criteria for bladder biopsies

This study included bladder biopsies for males and females within the age brackets of 6-65 years.

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3.4 Study sites

University of Ghana Medical School, Department of Pathology, Korle Bu Teaching Hospital has 4 sub-units: cytology unit, histopathology, immunology and molecular pathology. The catchment area of the Department is mainly in Southern Ghana. It serves the whole of Greater Accra including both public and private hospitals and clinics, the Eastern Region (Nsawam, Koforidua, Akwatia, Nkawkaw, Kwahu, Akuapim Mampong, Aburi, Krobo land), Tema (Zenu and environs), lower Volta, Cape Coast, Afia Nkwanta, Assin Fosu, Weija and environs (GaliIea, Dormeabra, Weija town)

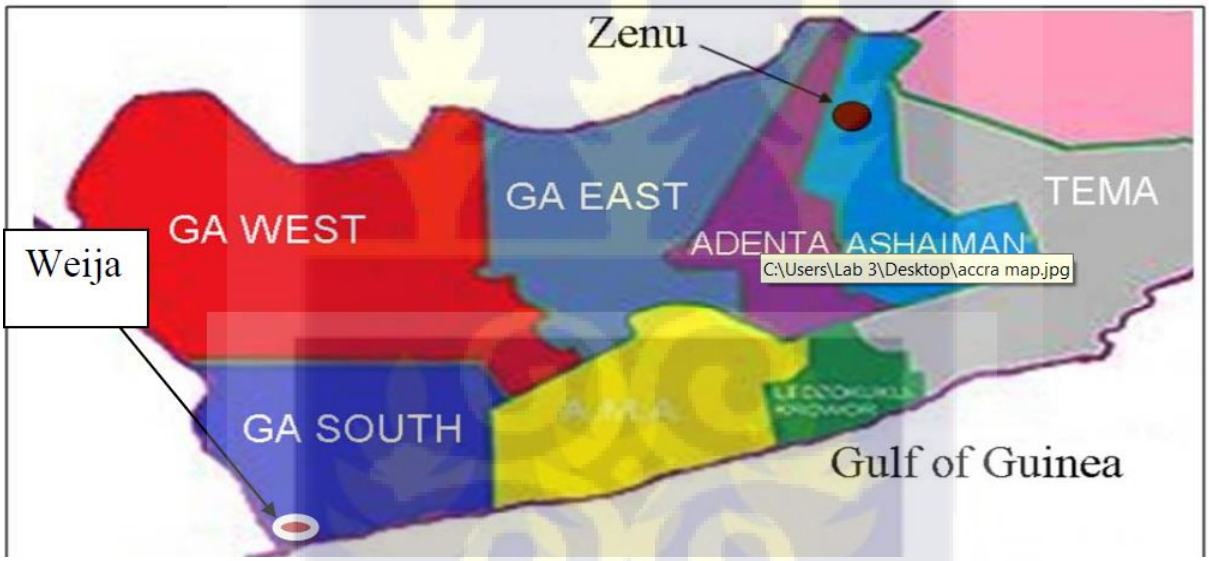

Figure 6: Map of southern Ghana showing Weija and Zenu as locations of the study sites

Source: Adapted and modified from Google maps (n.d.)

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3.4.1. Zenu community

Zenu popularly known as the Dam City is a suburb of Accra in the Greater Accra Region. It is situated at the Southern part of Ashaiman Municipality (Figure 5). This Municipality is located 30 km from Accra and 4 km to North of Tema. Ashaiman falls within longitude 0^0 01' latitude and 5^0 42' West. Ashaiman shares boundaries with North and East with Kpone Katamanso District and South and West with Tema Metropolis. Zenu has a total land area of 45 km^2. Its proximity to Accra and Tema makes it easy for community members to access schools, electric power, health facilities, major roads and markets. Most industrial workers are domiciled in Zenu due to the relative affordability of rent (GSS, 2014). Suitable soil and the presence of irrigation dam makes fishing and vegetable farming the main occupations of most people of Zenu. The lake created as a result of the dam is however infested with *S. haematobium*. There is a dam at Zenu that was originally constructed for irrigation of farmlands. The presence of snail species *Bulinus* and aquatic weeds at the shoreline of the dam together with stagnation of water and suitable climatic conditions of the water has made it suitable for *S. haematobium* endemic community. The proximity of the contaminated water to houses makes it convenient for household chores. Children use the contaminated water as recreational facility. Fish and crop farming are the main occupations of most males whilst most women are fish mongers. The frequency of contact with contaminated water by residents at Zenu, the absence of community hospital to treat urinary schistosomiasis, the absence of any active control program and lack of knowledge on transmission of *S. haematobium* makes it a suitable study site.

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3.4.2 Weija community

Weija serves as the administrative capital of Ga South district lying between South-Western part of Accra and bounded to South-East by Accra Metropolitan Area, North-East by Akwapim South, Ga West to the East, and to the north by West Akim, Awutu-Senya to the West, East to South- East by Awutu-Senya, South-West by Gomoa and Gulf of Guinea to the South (Figure 5). The total land area of Weija is about 341.838 km^2. It lies in dry equatorial climate zone, with annual average temperature ranging from 25.1^oC in August and 28.4 ^oC in February and March (Dickson *et al.*, 2001). Densu and Ponpon Rivers, drain into the Weija River. Although River Densu is infested with *S. haematobium*, River Densu serves more than half the population of Accra Metropolis. There are several small lakes created by these rivers in the community. The choice of Weija as a study site for this study was that it is *S. haematobium* endemic community which has been neglected for a long time.

There is a dam at Weija that was constructed as part of water treatment plant that serves people in southern Accra. The presence of snail species *Bulinus* and aquatic weeds at the shoreline of the dam together with the stagnation of the water and suitable temperature have made it suitable as a *S. haematobium* endemic community. Fish and crop farming are the main occupations of most males whilst most females are fish mongers. The frequency of contact with contaminated water by residents at Weija, the absence of community hospital to treat urinary schistosomiasis, the absence of any active control program and lack of knowledge on transmission and prevention of *S. haematobium* make it a suitable study site.

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3.5 Sample size estimation for community study

The sample size of 386 was calculated from the formula below

$$n = \frac{Z^2 \, x \, p(1-p)}{\varepsilon^2}$$

Where

z = the z score (1.96) at 95% CL

ε = the margin of error (0.05)

p = proportion (0.5)

n = the sample size

n= $\frac{1.96^{\,2} \text{ x } 0.5(1\text{-}0.5)}{0.05^2}$

n=386

3.6 Sample size estimation for bladder biopsies

The sample size of 386 was calculated from the formula below

$$n = \frac{Z^2 \, x \, p(1-p)}{\varepsilon^2}$$

Where

z = the z score (1.96) at 95% CL

ε = the margin of error (0.05)

p = proportion (0.5)

n = the sample size

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$n = \frac{1.96^2 \times 0.5(1-0.5)}{0.05^2}$

n=386

3.7. Sample size for cyclin D1 and HER2 proteins controls

Forty-two (42) of community urine sample had bladder pathology and presence of *S. haematobium* was used for cyclin D1 and HER2 so by simple proportion 13 was used as control for each marker as shown below.

Number of control = n/N x 100

n=42/551x100=12.5

n=13

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3.8 Questionnaire administration

With the use of standardized questionnaires (Appendix 5), consenting participants provided information on demographics, knowledge on preventive measures and transmission of schistosomiasis, symptoms of schistosomiasis. Any history of previous infection and type of treatment intervention received (herbal or orthodox), history of smoking and industrial waste exposure and any bladder issues on medication were sought for by the questionnaire to exclude those who have encountered confounders. After providing the required information, the study participants who met the criteria for the study were documented and given unique research numbers (alpha numeric) which identified them throughout the study. Child assent forms were given tochildren who could read and write to provide information on personal data. Study participants who needed assistance because of language barrier were helped by area coordinators recruited from the endemic communities to understand the project in order to give their informed consent at all study sites and subsequently included in the study.

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3.9. Community urine sample collection

Each study participant in the community was provided with clean wide mouthed leakproof urine container labelled with unique research number and was debriefed on what to do. Each study participant was told to agitate the bladder by doing a little bit of jogging or jumping to presumably dislodge the *S. haematobium* ova from the walls into the lumen of bladder before voiding the urine. This was to ensure the high probability of identifying *S. haematobium* ova in sediments of voided urine. On voiding the urine, each study participant was instructed to dispose of the first urine due to bacterial contamination and catch the terminal urine which was likely to contain the *S. haematobium* ova. Terminal urine was voided between the hours of 10:00-11:00 am when the excretion of ova was at its peak (Coulibaly *et al.*, 2018). The urine samples were immediately preserved on ice before transporting to the laboratory for analysis.

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3.10 Environmental Water Sample Collection

Samples of contaminated water was collected, pooled and preserved just as urine for CE analysis.

3.11 Laboratory work

Bladder pathologies on archived samples were confirmed by 2 pathologies using multi-header-microscope concurrently. Demographic data and other relevant data available were recorded on age, gender, clinical diagnosis, laboratory diagnosis and site of specimen. The data available on archived bladder biopsies were limited as compared to community sample. For laboratory procedures on community sample, people who gave consent to be part of the study and met the criteria, provided urine samples for the diagnoses of urinary schistosomiasis using parasitological technique and bladder pathologies using cytological technique. In the laboratory, after shaking gently, 30 ml of community urine sample of each participant was aliquoted and centrifuged to separate the fluid (supernatant) from the cellular (sediment) part. The supernatant was immediately transferred into a -80^0 freezer for the preservation of CE present as recommended by the reagent manual. The sediment was used for parasitological diagnosis of *S. haematobium* infection and cytological diagnosis of bladder pathologies. Enzyme-linked Immunosorbent Assay (ELISA) was used to analyse the concentration of COMT, the enzyme that metabolizes CE in urine to form semiquinone that is genotoxic to DNA. Genomic study was done on bladder pathologies and control to see if gene mutation was associated with CE. Genomic study was done to determine if CE was associated with gene mutation with subsequent production of corresponding protein in bladder pathologies due to urinary schistosomiasis.

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3.11.1 Parasitological examination of *Schistosoma haematobium* ova in urine sample

This section focuses on specific objective 1. For the community urine samples, macroscopy (visual inspection) was done to record colour, volume and clarity. Urine test strip was used to detect the presence of blood and recorded. The best method of diagnosing urinary schistosomiasis is to demonstrate the presence of *S. haematobium* ova in urine sediment quantitatively. The ova were concentrated to increase the sensitivity of the test.

Clear urine samples indicated the presence of few cells and cloudy urine samples indicated the presence of many cells. For clear urine samples, the distance between any 2 cells were far apart so during centrifugation at a higher speed, the cells would not knock each other to destroy the delicate cytoplasm so 1,500 revolution per minute (RPM) was used to separate cellular component (*S. haematobium* ova, red blood cells, inflammatory polymorphs, urothelial cells among other cells) from fluid component of the urine. For cloudy urine samples, a speed of 1,000 RPM was used to separate the cellular component from the fluid component because the cells were close to each other therefore the high likelihood of cells knocking each other and destroying delicate cytoplasm at a higher speed. For both clear and cloudy urine samples, the time of centrifugation was 5 minutes. The heavy materials like *S. haematobium* ova, red blood cells, inflammatory polymorphs and urothelial cells among other background cells that were present settled at the bottom round of the centrifuge tube. After centrifugation, the supernatant was decanted into another labelled storage tube and frozen at -80 ^{0} C for COMT analysis. The urine sediment at the bottom of the tube was mixed up well by first tapping gently the side of the tube and subsequently mixed up again using new Pasteur pipette. A volume of 54ul (1 drop) of urine sediment of each study

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participant was put on a labelled slide, covered with 24 x 24mm cover slip and examined using the Olympus light microscope (version CX41) for the presence of *S. haematobium* ova and RBCs. Those who were positive for urinary schistosomiasis were treated with praziquantel and advised not to get into contact with contaminated water to prevent re-infection with *Schistosoma* species

3.11.1.2. Parasitological examination for *S. haematobium* ova in archived sample by Haematoxylin and Eosin staining procedure

Addressing objective 1, the presence and count of *S. haematobium* ova was done on bladder biopsies. The sections were dewaxed/cleared using 2 changes of xylene for 2 minutes each. The smears were hydrated using descending grades of ethanol to water, 2 changes of absolute ethanol were used to remove the xylene from the section for 2 minutes each and descending grades (90%, 70%, 50%, and 30%) of ethanol to water. The sections were stained with Haematoxylin that stained the nuclei for 3 minutes and the blued using tap water after the pH (8.5) was checked, controlling blueing microscopically. The sections were afterward dehydrated using ascending grades of ethanol to absolute. The cytoplasm of the section was stained with Eosin for 2 minutes and excess stain was removed with absolute ethanol. As a precaution, sections were dried in oven for 10 minutes and cleared with xylene. The sections were cover slipped using DPX mountant.

3.11.2 Preparation of urine smear for S. haematobium ova count and cytopathology studies on community samples

This section focuses on specific objectives 1, 2 and 3 which was used to determine association between urinary schistosomiasis and bladder pathologies.

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For the cytopathology studies of cloudy and hazy urine sample of each study participant, 2 slides were labelled with unique research number and a drop (54 µl) of well mixed sediment of urine samples was put on labelled slide and a circular monolayer smear of 17 mm diameter was prepared from each drop. To ensure the smears were monolayered, a print was viewed underneath: if the print was blurry, it was suggestive of monolayer (Kim & Kim, 2014; Courtade-Saïdi *et al.*, 2016). For the clear urine samples indicating the presence of few cells, cytocentrifugation was done on the urine sediment using the cytospin. A perforated filter card (pore size 0.45 µm) were placed on the labelled gelatin-formaldehyde coated slides the right way The cytospin funnel was clipped to the labelled slide after mixing well, a drop of urine sediment (volume of 54 µl) of each urine sediment was put in funnel to prepare three of 7 mm each circular monolayer smear for each study participant (Sullivan *et al.*,2010). The importance of using the cytospin to prepare the smears in clear urine was to concentrate the few cells present in a small area for easy microscopic examination. The filter card absorbed the fluid in the urine whilst centrifugal force spread the cells in the urine sediment at low speed Whilst both conventional smears and cytospin smears were still wet, they were immediately immersed (wet fixation) in 95% ethanol for 15 minutes to preserve all cells (urothelial cells, RBCs, inflammatory polymorphs *S. haematobium* ova among other cells).

3.11.2.1 Cytological staining of cells of community samples

Smear of urine sediments were stained using Pap staining technique to provide contrast in epithelial cells for microscopy (Appendices 6 & 7).

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3.11.2.2 Pap Staining Technique

After 15 minutes of fixation, smears were hydrated using descending grades (95%, 70%, 50%, and 30%) of ethanol to water. The reason for the hydration was to precondition the cells to the next stain, Harris haematoxylin which is aqueous to prevent distortion of cells. The nuclei of cells were stained with Haematoxylin (pH-2.5) for 5 minutes and 'blued' afterwards using bluing agent by raising pH from 2.5 – 8.5, controlling bluing microscopically ensuring that the nuclei were blue. The essence of bluing was to strengthen the weak Van der Waal bond between the dye and phosphate in the nuclei. The nuclei have affinity for the hematin, the staining component in haematoxylin due to differences in changes and pH. Pap-stained urothelial cells bring out nuclei details of pathologic cells such as hyperchromasia, nuclear pleomorphism, coarse chromatin pattern, angulation of nuclear membrane, eccentric nuclei (in cells that under normal circumstances should have central nuclei), multinucleation, prominent nucleoli; the criteria upon which diagnosis of bladder pathologies are based on.

After bluing, the smears were dehydrated using ascending grades (30%, 50%, and 70 %) of ethanol up to 95% for 2 minutes each. The dehydrating process was to pre-condition cells to subsequent staining. Depending on components of cells cytoplasm, they had affinity for either Orange G 6 or EA both of which are cytoplasmic stains Orange Green (G) 6, the diluent been 95% ethanol. Cells containing keratin in cytoplasm was stained by Orange G 6. Excess Orange G 6 on slides was removed using 95% ethanol.

Cytoplasm of metabolically active epithelial cells without keratin were counterstained by Eosin Azzure. Cytoplasmic differentiation of various maturation levels of urothelial cells was made

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possible by Eosin Azzure stain because it contains different dyes (Eosin Y, light green). The basal and intermediate urothelial cell cytoplasm were stained by light green dye in shades of blue-green and superficial cells were stained by Eosin Y in shades of pink. Excess Eosin Azzure stain was removed using changes of 95% ethanol and absolute ethanol for 2 minutes each (Lerch *et al.*, 2019; Aslani, 1987).

As a precautionary measure, smears were air dried to prevent cloudiness when put in presiding solution, xylene which is not miscible with water. After drying smears, the refractive index (1.5) was improved bringing it close to that of glass by totally immersing smears in 2 changes in xylene for 2 minutes each. This stage also made cytoplasm of cells transparent by allowing light to pass through for effective morphological diagnosis. To protect the smear from scratch and damage, a permanent bound (mounting) was formed between glass slide and cover glass/slip by putting a drop of Distyrene plasticizer xylene (DPX) mountant at the area covered by the smears and appropriate sizes of coverslips were applied. It was ensured that the slides were wet with xylene and no air bubbles were trapped during mounting to prevent alterations in cells morphology (Saify & Tiwari, 2020). Slides were allowed to air dry overnight and was ready for microscopic examination.

The Pap stain technique is designed to achieve three purposes. The purpose is to give crisp (Placeholder1) nuclear detail for diagnosis of bladder pathologies using haematoxylin, cytoplasmic differentiation of epithelial (urothelial) cells since there are different maturation levels (superficial, intermediate, basal) using Orange Green (G) three as well as cytoplasmic transparency using xylene so that if cells overlap or covered by mucus or debris, light could pass through during microscopy.

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3.12 Parasitological examination for *S. haematobium* in archived biopsy and urine samples

Bladder pathologies were confirmed on archived H & E-stained bladder tissue section using multi-header-microscope concurrently.

Using Olympus microscope (version CX41), wet preparation and archived H & E-stained bladder tissue sections were examined for urinary schistosomiasis diagnosis using the battlement mode of slide examination (the examination of slides were done across the 24 cm length). The last portion of every previous field examined was added to the next so that nothing in smear was overlooked. Initially x10 objective lens was used to examine wet smears of urine sediment and findings confirmed using x40 objective lens. Parasitological diagnosis was done based on presence of *S. haematobium* ova with terminal spine in both urine wet preparation and H & E-stained bladder tissue. Absolute count of ova in wet preparations and H & E-stained bladder tissue were done. The presence of RBCs and polymorphs were recorded in wet preparations of urine sediments. Accidental findings of *Candida* species, bacterial infections were also recorded in wet preparations.

3. 13 Cytological and histopathological examination for diagnosis of bladder pathologies of community urine and archived biopsy samples

Using the microscope as above, Pap-stained smears of community sample for cytological diagnosis as well as archived H & E-stained bladder tissue sections were examined using the battlement method of slide examination. Initially x10 objective lens was used to examine smears of urine and tissues sections of bladder biopsies and subsequently x40 objective lens was used to confirm bladder pathologies.

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The bladder pathologies for the purpose of this study in community sample, were hyperkeratosis (HK), squamous metaplasia (SM), urothelial dysplasia (UD) and squamous cell carcinoma (SCC) all of which were epithelial in origin, a limitation to cytology. All nuclei were stained purple blue with Harris Haematoxylin. Cytoplasm of all urothelial cells stained had shades of pink, green-blue and blue depending on whether it is umbrella cell, intermediate or parabasal respectively with Eosin Azzure. Abnormal keratinization in bladder pathologies were stained Orange G 6 in Pap staining technique as seen in hyperkeratosis, keratinizing SM, keratinizing UD and keratinizing SCC (Botelho *et al.,* 2010; Khan *et al.,* 2002).

Histopathological diagnosis of bladder pathologies on archived H & E-stained bladder tissue section using the Olympus microscope was performed. The bladder pathologies for the purpose of this study, were hyperkeratosis (HK), squamous metaplasia (SM), urothelial dysplasia (UD) and squamous cell carcinoma (SCC), transitional cell carcinoma (TCC), urothelial cell carcinoma (UCC), all of which were epithelial in origin. In addition to the epithelial pathologies, subepithelial pathologies (cystitis glandularis, cystitis cystica, acute cystitis, chronic cystitis, leiomyoma) were also diagnosed, an added advantage to histopathological diagnoses.

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3.14 BIOMATIK Catechol-O-Methyltransferase (COMT) assay for catechol oestrogen metabolism (Appendix 8)

This section addresses Objective 4 which was to examine the relationship between catechol oestrogen levels in urine and the counts of *S. haematobium* ova. This technique also addresses specific objective 4 which was to assess the level of CE in bladder pathologies (Appendices 9-14).

3.14.1 Assay procedure for Catechol oestrogen metabolism using COMT

The number of ELISA Micro strips was determined and mapped taking into consideration the number of standards, controls and blanks needed. The 96 well plates pre-coated with specific anti-COMT were mapped and the first to seventh (1^{st} - 7^{th}) wells were allocated for serially diluted standards, the 8^{th} well was empty. The 9^{th} well was for blank reagent, the 10^{th} well was also empty to get correct readings for blank solution. The rest of the wells were for the study samples, contaminated water sample and controls. A 100 µl of the serial dilutions (10 ng/ml, 5 ng/ml. 2.5 ng/ml, 1.25 ng/ml, 0.625 ng/ml, 0.312 ng/ml, 0.156 ng.ml) of the COMT standard solution were pipetted into the first 7 wells, blank into 9^{th} well and samples and controls were pipetted into other wells. The plates were swirled, and plate sealer was used to cover the wells securely and incubated for 1 hour at 37^0 C. The plate sealer was removed, and excess fluid pipetted after 1-hour of incubation. Hundred (100) µl of detection reagent 'A' (Primary Antibody from which the serum antigen bounded to at one site) was added to each well. The wells were covered with plate sealer and incubated at 37^0 C for 1-hour. After incubation the solution was aspirated and 350ul of wash solution (BioTek 50TS, BioTek Instrument Incorporated, USA) was added to each well for 2 minutes before washing with the autowasher. Excess buffer was removed from the micro plates by snapping onto absorbent paper. The wells were

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totally washed 3 times as recommended by the instruction manual. After the last wash, the wash solution was decanted. The plate was decanted and blotted against absorbent paper.

A 100ul of detection reagent 'B' (Avidin conjugated to Horseradish Peroxidase HRP) working solution was added to each well, covered with a plate sealer and incubated for 30 minutes at 37^0 C. Five cycles of wash using 350ul washing solution added to each well, 2 minutes for each wash using automated 95-well micro plate washer (ELx 50.BioTeck Inc. USA). Wells were soaked with the buffer for 2 minutes, aspirated and blotted on an absorbent paper to remove excess buffer. The wells were further washed 4 times and after the last wash, all remaining last washing buffer was decanted, and plate inverted against absorbent blotting paper. A 90 µl of TMB (tetramethylbenzidine) substrate solution was added to each well, covered with a plate sealer and incubated for 30 minutes at 37^0 C with the microtiter plate protected from light. The solution in the wells was observed for blue coloration for standards and sample after incubation. A 50 µl of stop solution (2M sulphuric acid) was added to each well and the side of the plate was tapped gently to mix the solution thoroughly and colour changed to yellow (Appendix 17). Any drop of fluid and fingerprint was removed/cleaned from the underside of the plate. All bubbles were dislodged from plate. The assay was run in duplicates and average readings found.

3.14. 2 Reading of ODs and determination of concentration of COMT

Using the microtiter reader set at 450 nm, the absorbance was measured spectrophotometrically.

The optical density (OD) of each standard solution, control, samples, contaminated water from endemic areas and blank (zero standard) were made in duplications. An average OD of the seven standards was calculated. The average OD of blank readings was subtracted from each standard, control, and samples ODs. A standard curve was constructed by plotting the mean OD and

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concentration for each standard a best fit curve through the points on the graph or create a standard curve log-log graph paper with COMT concentration on the X-axis and absorbance on Y-axis.

3.14.3. Determination of Results

To determine the results, the average of the duplicate readings for each standard, control, and samples was found and the optical density of blank solution was subtracted. A standard curve (Figure 7) was plotted using the mean ODs and concentration for each standard and a best line of fit was drawn through the points on the graph to create a standard curve on log-log graph paper with COMT concentration on the x-axis (independent variable) and absorbance (dependent variable) on the y-axis. The concentration of COMT positively correlate with CE concentration. The parasitology was as above (specific objective 1) and absolute count of ova was done for every urine smear with the same quantity of urine and urine deposit.

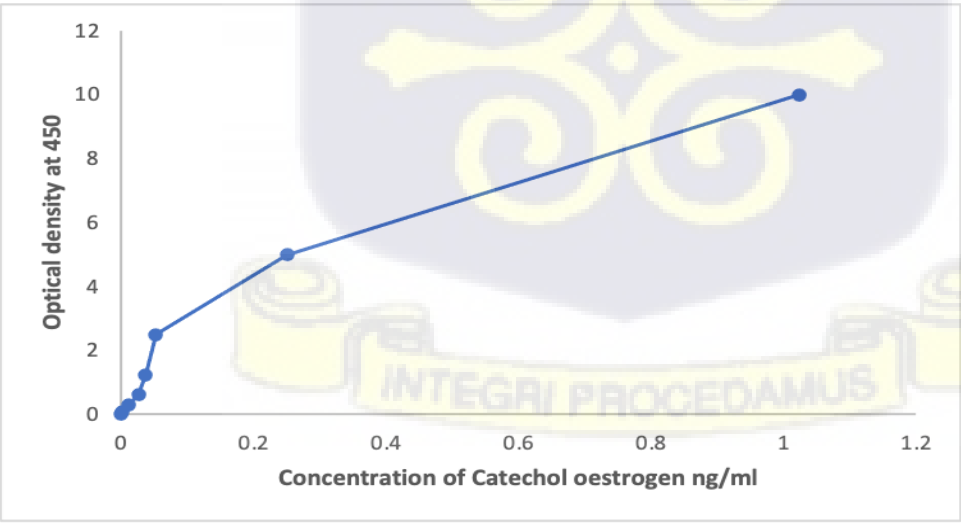

Figure 7. Standard curve for catechol oestrogen concentration

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3.15 Treatment of community participants diagnosed with urinary schistosomiasis

Depending on the body mass index, treatment was given to the study participants diagnosed with urinary schistosomiasis using praziquantel. Pre and post treatment levels were compared.

3.16 Genomic study

This section addresses specific objective 6 which was to assess the diagnostic performance of the markers used. Genetic study was done on both archived bladder biopsies and samples from the communities.

3.16 1 Procedure for Her 2 immunohistochemistry

Urine smears prepared as above were immunoassayed for HER 2 proteins. Positive HER2 tissue sections served as controls for the test. The tissue blocks of controls were put face down on ice and 3 µ thick of sections were cut using the rotary microtome. The sections were picked with forceps onto a perpex plate and flooded with 70% ethanol to help the tissue section float on water in a water bath set at 55 ^{0} C (10 ^{0} C below the melting point) of the paraffin wax used to embed the tissue to straighten the folds that may be trapped in the tissue sections. The serial sections were separated and 2 sections each were picked with labelled poly-L-Lysine coated slide for easy tissues adhesion onto

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slides. The slides were packed on a slide rack. Water was drained from the slides and slides put in oven set at 70 ^{0}C to melt the embedded wax for 30 minutes. Slides were put in 3 changes of xylene to deparaffinize the tissue section for 2 minutes each. The xylene was removed from the slides using 3 changes of absolute ethanol and rehydrated in graded ethanol to water. To deactivate endogenous peroxidase, positive control sections were further treated with 3% hydrogen peroxide for 10 minutes and slides washed with phosphate buffered saline (PBS) solution. Epitope retrieval solution was used to unmask HER2 antigen sites that might have been masked during tissue processing at 95 ^{0}C for 30 minutes. Slides were cooled down and washed with PBS for I minute. Blocking solution (Protein Block Serum: 0.25% Casein in PBS containing Carrier Protein and NaN3) was used for 5 minutes to prevent nonspecific immunostaining. Antigen retrieval step was omitted for the test (urine smears) because the procedure for smears preparation did not mask HER2 antigen. Using streptavidin-biotin peroxidase system, immunostaining was performed. The smears and sections were flooded with Biotinylated goat anti-rabbit antibodies (VENTANA (Lot: 187215) and slides were incubated in a wet chamber for 20 minutes at 37^0 C. Nuclei of cells in control sections and smears were stained with Mayer's Haematoxylin, dehydrated with absolute ethanol and mounted using DPX mountant.

3.16.2 Evaluation of immunohistochemistry and immunocytochemistry staining for HER2

The College of American Pathologists/ American Society of Clinical Oncology (CAP/ ASCO) scoring system was used to assess HER2 positivity. The level of HER2 was evaluated using membrane staining only by semi-quantitation and percentage of staining/intensity and scored on scale of 0-3+. Apart from scoring +3, all others were considered negative (>10% of stained cells were negative due to barely cytoplasm staining), +2 was weak to moderates staining (complete

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membrane staining in > 10% cells), +3 indicate strong and complete cytoplasm membrane staining (complete membrane staining in <10% cells).

3.16.3. Procedure for Cyclin D1 immunohistochemistry

Urine smear (test) and cyclin D1 positive control sections preparations were same as above. Antigen retrieval was done for the control sections as above and that step omitted for test for same reason as above. Urine smears were wet fixed in 95% ethanol for 2 hours. For both urine smears and cyclin D1 positive control sections, a 1/40 dilution of cyclin D1 antibodies (VENTANA LOT: F12294) were put on each slide and incubated at 37 ^{0} C for 2 hours. After incubation non-binding antibodies were washed 3 times with PBS for 5 minutes. The urine smears and cyclin D1 positive controls were incubated with blocking buffer solution in a moist chamber for 10 minutes at 37 ^{0} C. Afterwards, the smears and sections were flooded with Biotinylated goat anti-rabbit cyclin D1antibody (Lot: 210066) and incubated in a wet chamber for 20 minutes at 37 ^{0} C. Sections and smears were stained with Mayer's Haematoxylin, dehydrated with absolute ethanol and mounted using DPX mountant.

3.16.4 Evaluation of immunohistochemistry and immunocytochemistry staining for cyclin D1.

The College of American Pathologists/ American Society of Clinical Oncology (CAP/ ASCO) scoring system was used to assess cyclin D1 positivity. One cell stained positive in a field was scored as 1-25% of cells, 2 cells stained in a field was graded 26-50% of cells, 3 cells stained in a field was graded 51-75%, and 4 cells stained in a field was graded >75% of cells. The staining intensity was scored as 1 for mild staining, 2 for moderate staining and 3 for intense/strong staining. Multiplication of staining intensity and percentage score gives total score.

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3.16.5 Procedure for Ki67 immunocytochemistry

Poly-l—lysine coated slides labelled with research numbers were used to prepare circular monolayer smears of 9 mm diameter from urine sediment using the cytospin as above and smears immediately fixed wet in 95% ethanol for 15 minutes. Using the protocol of the kit and the Ventana machine, antigen of interest (Ki67) was re fixed in 95% ethanol for 1 hour. After wet fixation, the smears were incubated for 1 hour at 37^0 C. Immuno-stain (Ki67 antibodies + stains) was added to smears and incubated for 1 hour. Ki67 antigen receptors if present in the smear bounded to ligand of antibodies of Ki67. After incubation, the smears were washed in soapy water to get rid of oil that was used by the machine during the procedure. Smears were then dehydrated 3 times in absolute ethanol for 2 minutes each. The ethanol was cleared from smear using 3 changes of xylene for 2 minutes each. Finally, drops of DPX mountant were put manually on the smears on slide and covered with cover glass of size 24 x 24 mm.

3.16.6 Evaluation of immunohistochemistry and immunocytochemistry staining for Ki67

The College of American Pathologists/ American Society of Clinical Oncology (CAP/ ASCO) scoring system was used to assess Ki67 positivity (Appendices 18 A and 18 B). Twenty positive out of 100 stained cells viewed under the microscope was scored as 20% (Ki67 negative), 25 - 50 cells positive out of 100 cells stained was graded 25-50% (Ki67 positive),) and >70 cells positive out of 100 cells stained was graded ≥70% (positive for Ki67).

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3.17 Data analyses

All statistical evaluations were performed with statistical package for social science SPSS (version 17.0, IBM, Chicago, II, USA) for windows software. All data collected was analysed using descriptive analysis. Pearson chi-square, ANOVA and odds ratio were used to test the association between urinary schistosomiasis and bladder pathologies. Spearman correlation was used to examine the relationship between catechol oestrogen levels in urine and quantity of *S. haematobium* ova. ANOVA and Tukey multiple comparison were used to assess the difference in mean of levels of CE in different bladder pathologies (assess the correlation between catechol oestrogen and bladder pathology). To determine diagnostic performance of molecular marker associated with bladder pathologies, ROC curve was plotted to determine which of the markers was sensitive and hence, can be used for bladder cancer surveillance in communities where urinary schistosomiasis is endemic.

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3.12 Archived bladder biopsies

Data on bladder pathologies with the presence of *S. haematobium*, diagnosed over a period of ten years (2009-2019) were retrieved from the archives of Department of Pathology using stratification (every third) and analysed.

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CHAPTER FOUR

4.0 RESULTS

4.1. Characteristics of study Participants and Samples

4.1.1 Archived Samples

Out of the 551 bladder biopsies that were selected by stratification (every third), 269 (48. 8%) were from female participants. Thirteen (2.3%) of the entire samples were from volunteers aged from 6-19 years, with 242 (43.9%) coming from participants aged 60 years and above (60-69 years old) (**Table 1**). The overall mean age was 51.85 years and the median age, 54 years (IQR 41.0- 65.0). The mean age of females was 49.36 years and median age is 51 years (IQR 40.0- 62.0). The mean age of males was 49.80 years and median age is 54 years (IQR 40.0- 63.0). The clinical history available on request cards were 150 (27.2%) haematuria, 13 (2.3%) dysuria, 3 (0.5%) urine incontinence, 13 (2.3%) urine retention, 7 (1.3%) Urinary Tract Infection, 16 (2.9%) urinary schistosomiasis and 2 (0.4%) bladder cancers. One-hundred and eight (19.6%) of the bladder biopsies had *S. haematobium* ova in the stained slides

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Table 1: Age and sex distribution of bladder pathologies in archived biopsy samples of patients reporting at the Korle Bu Teaching Hospital (KBTH) over a period of 10 years (2009-2019)

Variables	Bladder Pathologies		
	Total number tested	Number Positive (%)	Chi-square (P-value)
Age Group (years)			
6-19	13	3 (23.1)	25.010
20-29	45	6 (13.3)	**(<0.001)**
30-39	57	26 (45.6)	
40-49	92	42 (45.7)	
50-59	102	51 (50)	
60+	242	123 (50.8)	
Sex			
Female	269	135 (50.2)	4.547
Male	282	116 (41.1)	**(0.033**

+fishers exact p-value (some cells frequencies < 5)

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4.1.2 Community urine Samples

The number of people recruited into the study from the communities were 450. Out of the 450 people, five were excluded because they encountered confounders, whereas 14 withdrew from the study. Four hundred and thirty-one (431) study participants aged between 6-65 years took part in the study. The overall mean age was 27.1 years and the overall median age was 23.0 (IQR 13.0-38.0). The mean and median ages for male study participants were 26.2 and 22 years, respectively. The mean and median ages for females were 28 and 24.5 years, respectively.

There was signimficant difference among age groups regarding *S. haematobium* infection (p =0.041). Two-hundred and twenty-seven (52.7%) of the study participants were males. *S. haematobium* infection rate was significant higher in male (50.2%) and female (41.1%) respectively (p=0.027).

Majority (340) of study participants representing 78.8% had primary education. People with occupational exposure to risk of infection (fisherman/farmers/fishmongers) were 104 (24.1%) (Table 2). Majority 327 (75.8%) of study participants were from Zenu and its environs (p= 0.001) (Table 3). Majority 377 (87.4) of the study participants had contact with the contaminated water body. Majority 259 (60.1%) had lived in the endemic communities 1-5 years. Minority 56 (12.9%) had a history *S. haematobium* infection and 34 (7.8%) had a history of treatment of schistosomiasis two years and above. Minority 156 (36.1%) had no knowledge on transmission of *S. haematobium* (bilharzia) infection whilst majority 142 (32.9%) also had no knowledge on prevention of S. *haematobium* infection. The overall *S. haematobium* prevalence was 9.7% (42), Thirty-nine (9.04%) people were diagnosed with bladder pathologies, One-hundred and 17 (27.1%) had experienced blood in urine (haematuria) at least once in their life in

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the endemic community. Minority 53 (12.2 %) of study participants had blood in their urine sample microscopically; four were frank haematuria at the macro level. There was significance association between the presence of RBCs, and *S. haematobium* infection. Forty (9.2%) of study participants presented with urine sample with appearance collectively indicating some disease state of bladder. There was significant association between colour of urine, and *S. haematobium* infection (Table 3). Thirty -two (82.1%) out of the 39 study participants diagnosed with bladder pathologies has *S. haematobium* infection as well.

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Table2: Demographics data on community participants

Factors	Number	Percentage (%)
Age, In years		
6-19	178	41.2
20-29	87	20.18
30-39	60	13.92
40-49	40	9.28
50-59	32	7.42
≥ 60	30	6.96
Sex of respondent		
Male	227	52.70
Female	204	47.30
Current occupation		
Farmer/fisherman/fishmonger	104	24.12
Student	187	43.30
Other profession	140	32.40
Educational Level		
Primary	340	78.88
Secondary	56	12.99
Tertiary	35	8.12
Location		
Zenu	327	75.80
Weija	104	24.10
Had contact with a dam		
No	54	12.50
Yes	377	87.40
Sources of drinking water		
Pipe	281	65.10
Dam	150	34.80
No. of Years living in community		
1-5	259	60.00
6-10	101	23.40
11 years and above	71	16.40
History of *S. haematobium* infection		
No	375	87.00
Yes	56	12.90
History of treatment for *S. haematobium* for last 2 years		
No	397	92.10
Yes	34	7. 8

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Knowledge of bilharzia transmission		
No	275	63.80
Yes	156	36.20
Knowledge of bilharzia prevention		
No	289	67.00
Yes	142	32.90
Presence of *S.h*		
No	389	90.20
Yes	42	9.70
Bladder pathology		
No	392	90.95
Yes	39	9.04
Bladder pathology with *S. h*. infection		
No	399	92.57
Yes	32	7.42
Experienced blood in urine		
No	314	72.80
Yes	117	27.10
Presence of Red Blood Cell in urine		
No	378	87.60
Yes	53	12.20
Colour of urine		
Blood stained	4	0.9.2
Hazy	4	0.92
Hazy straw	5	1.16
clear straw	391	90.9
cloudy straw	27	6.20
Urinary Tract Infection (UTI)		
No	405	93.90
Yes	26	6.03
Candida		
No	416	96.5
Yes	15	3.40
Age, In years	Median(overall)=23.0 (IQR 13.0-38.0)	
Age, In years	Mean (overall) =27.1(IQR 13.0-38.0)	
Age in years	Mean (male)=26.2	
Age in years	Median (male) =22	
Age in years	Mean (female)=28.0	
Age in years	Median female =24.5	

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Table 3: Age and sex distribution of bladder pathologies among community participants using urine samples

Variables	Bladder Pathologies		
	Total number tested	Number Positive (%)	Chi-square (P-value)
Age Group (years)			
6-19	178	20 (11.2)	8.26((0.0410)
20-29	87	8(9.1)	
30-39	64	10 (15.6)	
40-49	40	2 (5.0)	
50-59	32	3 (9.3)	
60+	30	0(0.0)	
Sex			
Female	204	12 (6.8)	4.92((0.027)
Male	227	30(13.2)	

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4.1.3 Association of bladder pathologies with age and sex

With the patients archived biopsy samples, analysis showed that the prevalence rates of bladder pathologies varied significantly among the age groups with older groups having higher rates (χ^2 =25.010; p<**0.001**). Significant variations were also observed among the age groups of the community urine samples, with younger age having higher rates but lesser grades of the disease (X^2=8.260, p=0.0410) (Table 4). Whereas prevalence of bladder pathologies was high among females, with the archived biopsy samples (χ^2 =4.547; p<**0.033)** (Table 1 above**),** in the community samples however**,** males had a higher prevalence than females (X^2=4.920, p=0.027) (Table 3 above).

A Logistic Regression Model was fitted to explain the relationship between the dependent variable (bladder pathologies) and the explanatory variables (sex and age group). Females were found to be 1.44 times more likely to test positive for bladder pathologies as compared to males based on the archived samples [OR = 1.442, (95% CI: 1.029, 2.019) **p=0.033**] (Table 4). Also, males were 41% (0.41times) less likely to develop bladder pathologies than females regarding the community samples (OR=0.410, (95% CI: 0.204, 0.825) **p=0.0124**) (Table 5). These analyses showed that females were more likely to develop bladder pathologies (Appendices 16 A1, A2, 16 B) than males.

Also, a logistic regression analysis showed patients aged 20-29 years were 85% (8.5 times) less likely to test positive for bladder pathologies as compared to those patients aged 60 years and above, based on the archived samples [OR = 0.149, (95% CI: 0.061, 0.365) **p<0.001**] (Table 4). With regard to the community samples, the analysis showed that participants aged between 6-19 years were 1.76 times more likely to have bladder pathologies with reference to those above 60 whereas males were 41% less likely to develop bladder pathologies than females (OR=0.410, (95%CI; 0.204, 0.825) **p=0.0124**) (Table 5)

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Table 4: Age and Sex, and the Odds of Having Bladder Pathologies in patients with archived biopsies

Variables	Estimate	SE	Odds Ratio	95% CI	P-value
Age Group (years)					
60+	**Ref**				
6-19	-1.237	0.195	0.290	0.078, 1.081	0.065
20-29	-1.905	0.068	0.149	0.068, 0.365	**0.000**
30-39	-0.209	0.240	0.811	0.455, 1.448	0.479
40-49	-0.207	0.200	0.813	0.502, 1.315	0.398
50-59	-0.033	0.228	0.967	0.609, 1.537	0.889
Sex					
Male	**Ref**				
Female	0.366	0.248	1.442	1.029, 2.019	**0.033**

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Table 5: Age and Sex, and the Odds of Having Bladder Pathologies among community participants using urine

Variables	Estimate	SE	Odds Ratio	95% CI	P-value
Age Group (years)					
60+	**Ref**				
6-19	-2.103	0.282	1.76	1.039-2.982	**0.017**
20-29	-0.393	0.311	1.16	0.542-2.508	0.346
30-39	-1.480	0.370	1.80	0.826-3.953	0.069
40-49	-0.718	0.480	0.58	0.135-2.527	0.236
50-59	-0.427	0.707	0.639	0.083-4.958	0.334
Sex					
Male	**Ref**				
Female	0.207	1.417	0.410	0.204-0.825	**0.0124**

4.2 Prevalence of *Schistosoma haematobium* infection from study samples

Prevalence of *S. haematobium* infection generally varied among the age groups of the patients with archived bladder biopsy samples (χ^2=59.76, p<0.001) and decreased with age, however, it did not vary significantly among the age groups of the community participants (χ^2=9.02, p=0.1083) but also decreased with older age. As expected, the prevalence of S. *haematobium* infection for all age groups as well as the overall were significantly higher in the patients with archived biopsy samples than in the community participants' urine samples (χ^2=14.44, **p=0.0130**). Whereas participants within the 20-59 age brackets of the patients with archived biopsy samples had the highest prevalence, the age groups with highest infection rate for the participant from the community were under 20, 20-29 and

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30-39 years. The prevalence of S. *haematobium* infection was higher in males for community participants (χ^2=4.73, p=**0.0296**) (13.2%) but did not differ significantly in the patients archived bladder biopsy samples (χ^2=2.29, P=0.1302).

The prevalence was also higher at Weija and its environs (19.2%) than Zenu and its environs (9.7 %.) (χ^2=14.02, **p=0.0002**) (Table 6).

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Table 6: Prevalence of *Schistosoma haematobium* from urine of community study participants and archived biopsy samples from patients diagnosed with bladder pathologies

Age	Archived biopsy Samples		Community urine Samples	
	Number tested	Number Infected (%)	Number tested	Number Infected (%)
6-19	13	2 (15.4)	178	20(11.2)
20-29	39	18 (46.2)	87	8(9.1)
30-39	51	17 (33.3)	64	10 (15.6)
40-49	94	31 (33.3)	40	2 (5.0)
50-59	101	18 (17.8)	32	1 (3.1)
60+	255	19 (7.5)	30	0 (0.0)
Sex				
Female	266	44 (16.5)	204	14 (6.8)
Male	282	61 (21.6)	227	30 (13.2)
Community				
Zenu & environs		-	327	22(6.7)
Weija& environs		-	104	20 (19.2)
Overall	551	105 (19.1)	431	42 (9.7)

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4.3 Association between urinary schistosomiasis and bladder pathologies

A Pearson's chi-square test or Fisher's Exact test also established a statistically significant association between some bladder pathologies and testing positive or negative for *S. haematobium* infection. There was a significant association between squamous cell carcinoma (Appendix 16a, A1) ($\chi^2 = 56.738$, **p<0.001**), Transitional cell carcinoma ($\chi^2=13.352$, **p<0.001**), Chronic cystitis ($\chi^2=6.811$, **p=0.009**) and schistosomiasis infection (Negative/Positive) (Table 7).

Again, A Logistic Regression Model was fitted to examine the relationship between *S. haematobium* infection among patients who were diagnosed with squamous cell carcinoma (SCC), transitional cell carcinoma (TCC) and chronic cystitis (CC). Patients who were diagnosed with squamous cell carcinoma were 6.8 times more likely to test positive for *S. haematobium* infection as compared to those patients who tested negative for the disease [OR = 6.833, (96% CI: 3.944 to 11.839) **p<0.001**]. Patients who were diagnosed with transitional cell carcinoma were 64% less likely to test positive for schistosomiasis as compared to those who tested negative for the disease [OR = 0.358, (95% CI: 0.203 to 0.632) **p<0.001**]. Also, patients who were diagnosed with chronic cystitis were 55% less likely to test positive for schistosomiasis as compared to those who tested negative for chronic cystitis [OR= 0.445, (95% CI: 0.239 to 0.828) **p=0.011]** (Table 8)

ANOVA of egg counts revealed high significant differences among the categories (**p<0.0001**). In pairwise comparisons, the mean egg counts of controls were significantly lower than those of all the pathology groups {Control vs HK: Mean difference (95% CI): 1.45 (0.41 to 2.49), **p=0.0026**; Control vs SM: 4.85 (3.81 to 5.89); **p<0.0001**; control vs UD: 8.48 (7.23 to 9.72), **p<0.0001**}. Comparisons between pathologies also showed highly significant variations for all pairs

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(**p<0.0001**) with urothelial dysplasia (Appendix 16a A2) having the highest counts and HK the least (Figure 7)

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Table 7: Association between Bladder Pathologies and S. haematobium infection

Variables	Uninfected (%)	Infected (%)	χ^2 (P-value)
Squamous Metaplasia			
Negative	431 (80.4)	105 (19.6)	2.18
Positive	9 (100)	0 (0)	(0.218)
Acute Cystitis			
Negative	426 (80.4)	104 (19.6)	1.57
Positive	14 (93.3)	1 (6.7)	(0.324)
Squamous cell carcinoma			
Negative	410 (85.4)	70 (14.6)	56.73
Positive	30 (46.2)	35 (53.8)	**(<0.001**
Transitional cell carcinoma			
Negative	293 (76.7)	89 (23.3)	13.35
Positive	147 (90.2)	16 (9.8)	**(<0.001**
Urothelial Dysplasia			
Negative	438 (81)	103 (19)	2.44
Positive	2 (50)	2 (50)	(0.169)
Chronic Cystitis			
Negative	334 (78.4)	92 (21.6)	6.81
Positive	106 (89.1)	13 (10.9)	**(0.009**
Urothelial cell carcinoma			
Negative	436 (80.6)	105 (19.4)	0.962
Positive	4 (100)	0 (0)	(1.000)+
Hyperkeratosis			
Negative	6(33.3)	9(66.6)	38.2
Positive	377(92.0)	33(28.2)	< 0.0000

+Fishers exact p-value (some cells frequencies < 5)

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Table 8: S. haematobium infection and the Odds of Having Bladder Pathology

Variables	Estimate	SE	Odds Ratio	95% CI	P-value
Squamous cell carcinoma					
Negative	**Ref**				
Positive	1.922	1.916	6.833	3.944, 11.839	**<0.001**
Transitional cell carcinoma					
Negative	**Ref**				
Positive	-1.026	0.104	0.358	0.203, 0.632	**<0.001**
Chronic Cystitis					
Negative	**Ref**				
Positive	-0.809	0.141	0.445	0.239, 0.828	**0.011**

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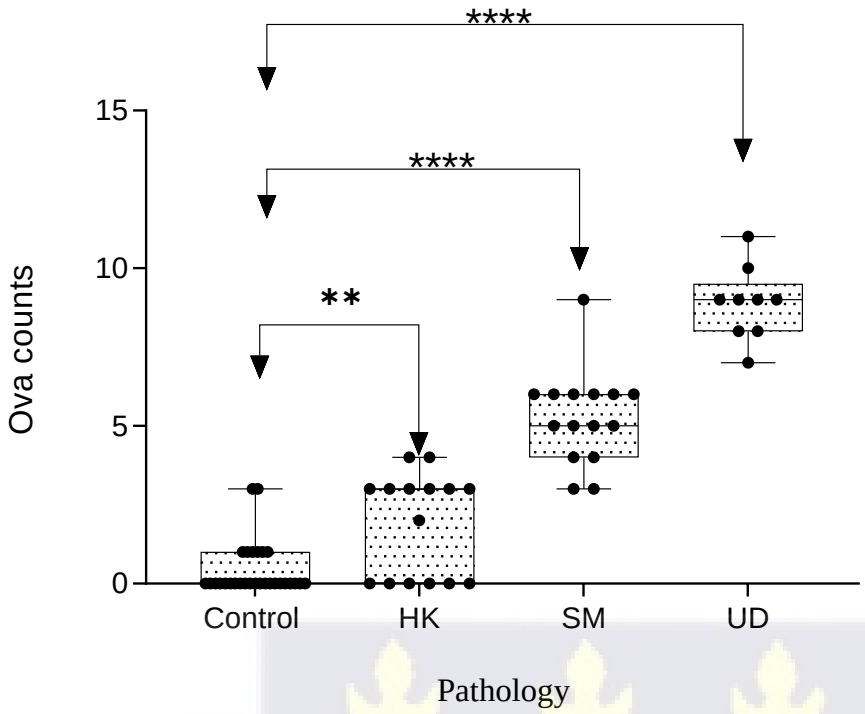

Figure 8: Comparison of mean ova counts among bladder pathologies. HK, SM and UD represent hyperkeratosis, squamous cell carcinoma, and urothelial dysplasia. The whiskers denote 5% and 95% percentiles

4.4 Relationship between catechol oestrogen levels in urine and the quantity of *S. haematobium* ova

A spearman correlation test of *S. haematobium* ova counts against concentration of CE revealed a weak but highly significant correlation between ova counts and CE concentration in urine (r=0.4218, 95% CI: 0.3379 to 0.4991, **p<0.0001)** (Figure 8).

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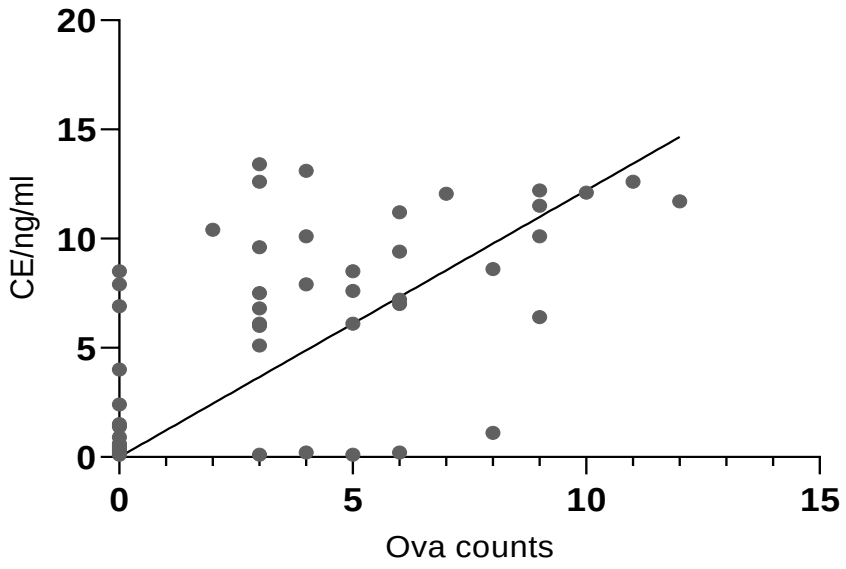

Figure 9: Spearman correlation between S. haematobium ova count and concentration of CE

4.4.1 Comparison of pre and post treatment levels of catechol oestrogens

Comparison between pre and post treatment levels of catechol oestrogen in urine of community participants was estimated the following was observed. The mean difference of catechol oestrogen levels between 1st week post treatment of urinary schistosomiasis and pre - treatment of urinary schistosomiasis was -2.672. (CI = -5.349-0.0040; p<0.051;)

The mean difference between catechol oestrogen levels 3rd week post treatment and pre-treatment of urinary schistosomiasis was -3.710. (CI = -6.4128 - -1.0085; p< 0 .001;)

The mean difference between catechol oestrogen levels 4th week post treatment and pre-treatment of urinary schistosomiasis was -4.750, CI (-7.4269- -2.0738; p< 0.001) (Figure 9)

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4.5. Concentration of CE among Bladder Pathologies

The concentration of CE in urine of participants with HK, SM, UD and controls ranged between 0.100 ng/ml to 13.40 ng/ml, 0.100 ng/ml to 11.20 ng/ml, 1.100 to 12.60 ng/ml, 0.100-6.900 ng/ml respectively. The mean CE in HK, SM, UD and controls were 8.460 ng/ml, 6.320 ng/ml, 9.628ng/ml and 0.2462 ng/ml, respectively. ANOVA of mean concentration of CE revealed high significant differences among the bladder pathologies (**p<0.0001**) with the difference increasing with severity of bladder pathologies. In pairwise comparisons, the mean CE concentration of controls was significantly lower than those of all the pathologies (**P<0.001**). The mean concentration of CE of UD and HK were also significantly higher than that of SM (p<0.001)

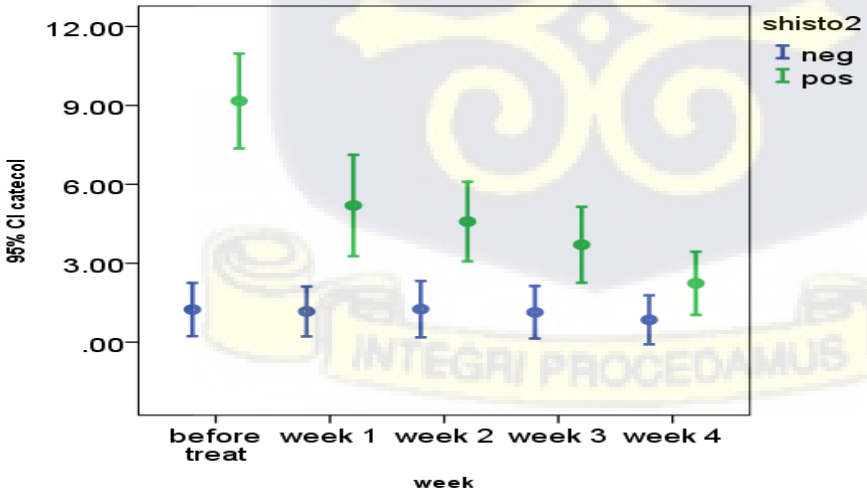

Figure 10: Post treatment concentration of catechol in urine

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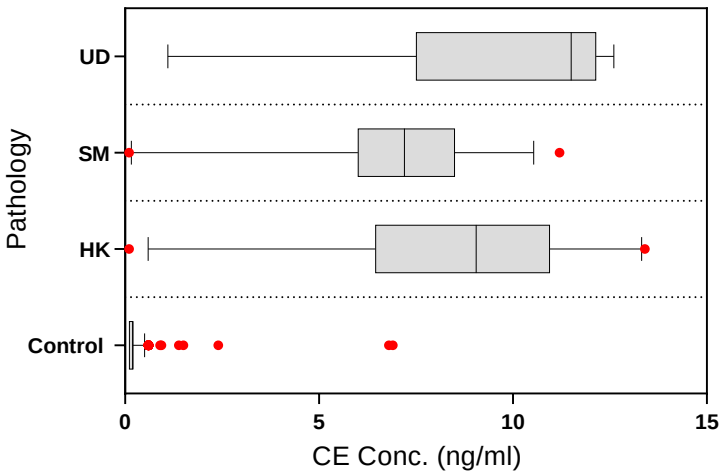

Figure 11: Comparison of concentration of CE in bladder pathologies. HK, SM and UD represent hyperkeratosis, squamous cell carcinoma, and urothelial dysplasia. The whiskers denote 5% and 95% percentiles.

Table 9: Tukey's multiple comparisons test of CE

Comparisons	Mean Diff.	95 % CI of diff.	P-value
Control vs. HK	8.214	7.285 to 9.142	<0.0001
Control vs. SM	6.074	5.311 to 6.837	<0.0001
Control vs. UD	9.382	8.404 to 10.360	<0.0001
HK vs. SM	2.140	0.957 to 3.323	<0.0001
HK vs. UD	1.168	-0.164 to 2.499	0.1087
SM vs. UD	3.308	2.086 to 4.530	<0.0001

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4.6 Diagnostic performance of catechol oestrogen as biomarkers in urine for detection of bladder pathologies

To find out if CE could be used as a biomarker for diagnosing bladder pathologies in urine, Receiver Operating Characteristic (ROC) curves were drawn as shown in Figure 10. These curves and the corresponding area under the curves (AUCs) show that CE has a very good predictive ability to discriminate bladder pathology patients from normal individuals. The AUC for all bladder pathologies put together was 0.88 (95% CI: 0.80 to 0.95, $p<0.0001$). Those of HK only and SM were 0.84 (95% CI: 0.73 to 0.96, $p<0.0001$) and 0.78 95% CI: 0.62 to 0.93, $p=0.0003$), respectively (Figure 12).

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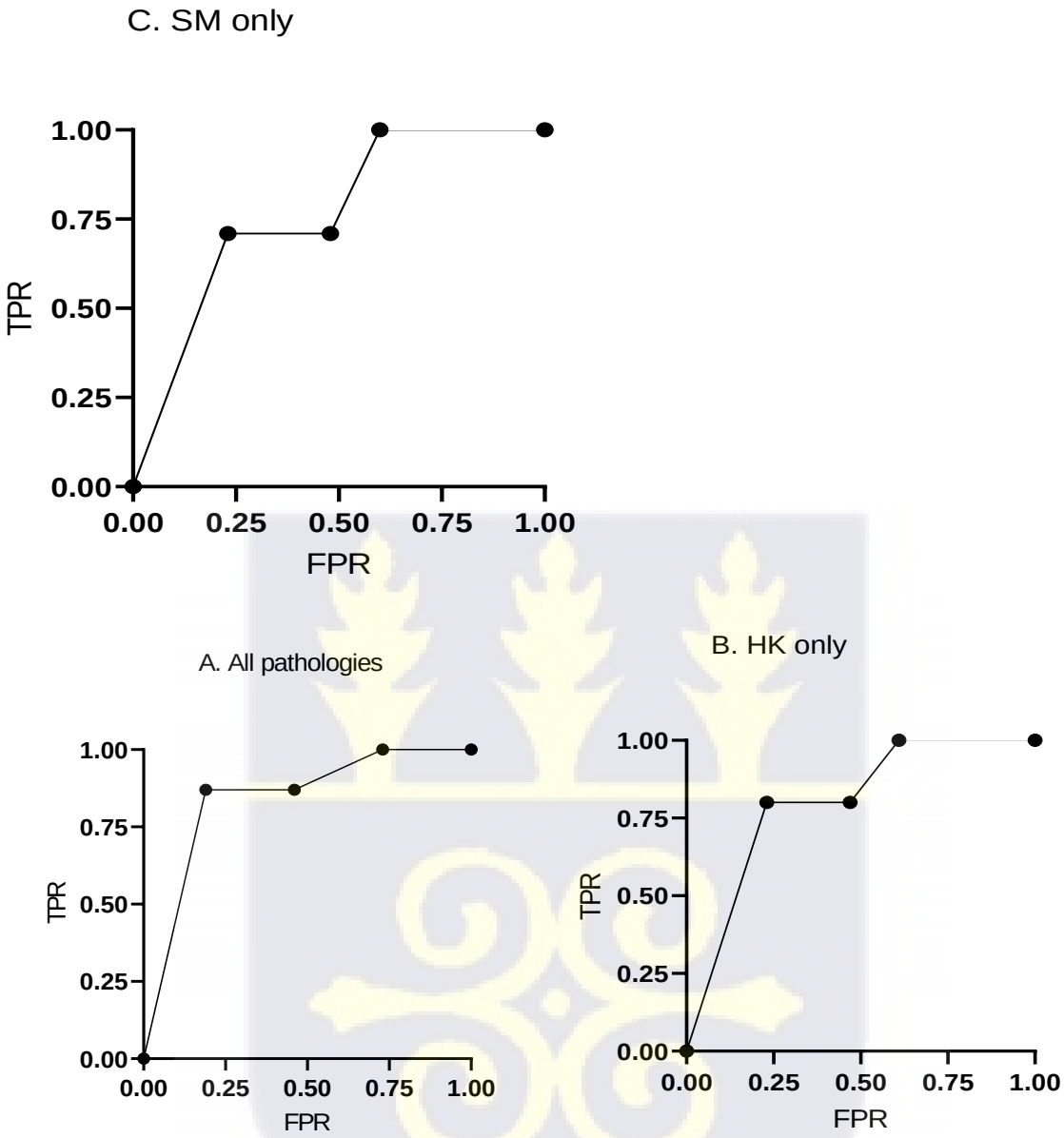

Figure 12: Catechol Oestrogen (CE) ROC curves of pathologies. A, B and C represent the curves for all pathologies put together, squamous metaplasia and hyperkeratosis, respectively. TPR and FPR represent true and false positive rates, respectively

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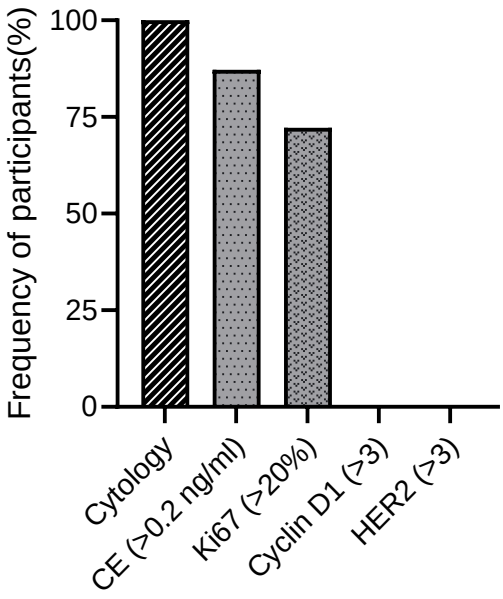

Figure 13: Positive rate with molecular markers compared with cytology. CE represents catechol oestrogen. Diagnosis of bladder pathologies were based on CE level>0.2 ng/ml, Ki67 (>20%), meaning more than 20% of cells; cyclin D1>3 and HER2>3.

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CHAPTER FIVE

5.0. Discussion

Schistosoma haematobium, the causative agent of urinary schistosomiasis, accounts for about 65% of all schistosomal diseases worldwide. The disease is also endemic at Zenu and Weija communities in Greater Accra Region of Ghana (Tetteh-Quarcoo *et al.*, 2020; Anyan *et al.*, 2019;;Amoah *et al.*, 2018; Rashid *et al.*, 2011; Dent & King, 2007; Smith & Christie, 1986;). Prevalence of bladder pathologies is high in *S. haematobium* endemic communities worldwide (Arora *et al.*, 2019). Preventive treatment of urinary schistosomiasis has resulted in a reduction in bladder pathologies suggesting a strong association between urinary schistosomiasis and bladder pathologies (Botelho *et al.*, 2013; Rambau *et al.*, 2013). In Egypt as well as other countries where schistosomiasis is endemic, over 25% of all reported cancer cases are *S. haematobium*-associated bladder cancers (Khaled, 2013; Elsebai, 1977). In Ghana review study of surgical bladder specimen demonstrated involvement of *S. haematobium* in bladder pathologies at the Korle Bu Teaching Hospital (Der *et al.*, 2015). Bladder pathologies due to urinary schistosomiasis starts as sub-urothelial lesion and later involve the epithelium all of which can be diagnosed by histopathological methods but the procedure for sampling is invasive, making histopathology inappropriate as a screening tool. Urine cytology has an advantage over histopathology as a screening tool but has a limitation due to the fact that sub-epithelial bladder pathologies cannot be picked because the cells do not shed into voided urine. (Giordano *et al.*, 2019 Ahmed *et al.*, 2014; Botello *et al.*, 2011; Tzanetou *et al.*, 2007). Since cytological diagnosis of subepithelial bladder pathology in urinary schistosomiasis can be missed when the lesion is focal and with minimal shedding into urine or if it is subepithelial, it will be helpful to identify another prognostic biomarker in urine to screen for those who are at risk of developing bladder pathology in *S. haematobium* endemic communities (Adebayo *et al.*, 2018; Cavalieri & Rogan, 2006; Planz *et al.*, 2005).

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The *Schistosoma* egg antigen (SEA) containing CE is hypothesized to be associated with bladder pathologies in urinary schistosomiasis. Some cell cycle genes like cyclin D1 as well as HER2 and Ki67 proteins are over expressed in bladder pathologies. In this study, there was a wide variation in age of study participants. This was to capture bladder pathologies due to urinary schistosomiasis in all age groups so that the sampling would be representative. Understanding of the prevalence of urinary schistosomiasis infection and geographical distribution of the disease in different endemic settings is very critical in the planning and implementation of control, elimination and eradication programs.

The prevalence of S. *haematobium* infection generally decreased with age among community study participants. The high prevalence of *S. haematobium* infection among youngest age brackets could be due to frequent contact with contaminated water for recreational purpose and commuting along water bodies to and from school, the proximity of their houses to the contaminated water body making it accessible for domestic use, fish and crop farming and fish mongering, recreation, lack of knowledge, economic) and demographic factors associated lack of immunity against *S. haematobium* infection as supported by the questionnaire study on knowledge-attitude-behaviour-practice.

S. haematobium infection rate was significantly higher in males than females among the community study participants. The high prevalence of *S. haematobium* infection in males could be attributed to exposure to more risk factors such as fish and crop farming and fish mongering, recreation, lack of knowledge, economic and demographic factors associated with infection hence they are having frequent contact with contaminated water. The prevalence however did not differ significantly in archived samples because both males and females were sick and presented with symptoms to the hospital. In a similar study by Ismail *et al.* (2014) and Degarage *et al.*, (2015), using archived samples, there was no variation in prevalence of *S. haematobium* in terms of gender. The prevalence

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was also higher at Weija and its environs (19.2%) than Zenu and its environs. The geographical variation in prevalence of *S. haematobium* infection between Zenu and Weija could be due to neglect of endemic communities in terms of control of the disease. There has been no Mass Drug Administration (MDA) at Weija for the past 10 years, whereas shorelines of the Weija dam continue to harbour more of intermediate snail host (*Bulinus* species) and abundant weeds serving as habitat for the snails at the shorelines. The water body at Weija is more stagnant facilitating the aquatic life cycle. Residents also dispose of urine and stool samples into the water during fishing, which contributes to the persistent reinfection after treatment.

Although old age is associated with weak immune system, the prevalence of *S. haematobium* infection declined with age in the community samples could be due to decreases in water contact hence reduced parasite burden The prevalence was significant among age group 6-19 years with the community urine sample. This could be attributed to one or more factors such as exposure risk/increased out-door activity, immunity not well developed, malnutrition and scarcity of pipe borne water. Similar studies in Egypt, Tunisia, Nigeria revealed that age of development urinary schistosomiasis was lower **(**Onile *et al.*, 2016; Khaled, 2013; Elsebai, 1977).

Prevalence in of *S. haematobium* in patients archived samples was significant because there could be

selection bias of sick people, healthcare seeking behavior, diagnostic testing as well as referral bias.

The rate of knowledge on transmission of *S. haematobium* was higher than the rate of knowledge on prevention (avoiding water contact, MDA) of the disease. The deficit in knowledge on prevention might have contributed to contamination of water by those having the infection and aiding transmission to those without infection. The presence of RBCs, chylous, cloudy as well as haziness

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of urine were predictors of *S. haematobium* infection in the community samples because the appearance of urine gives an indication of bladder health.

Significant variations were observed in the prevalence rates of bladder pathologies among the age groups of both the archived and the community participants. The younger age groups had higher rates of bladder pathologies among the community participants but with lesser grades of the disease. This is in consonance with other studies suggesting bladder pathology developing early at younger age in urinary schistosomiasis (Aula *et al.*, 2021; Rambau *et al.*, 2013). Some research findings in Ghana, Egypt, Tunisia, Nigeria where age of development of bladder pathology due to urinary schistosomiasis was lower than industrial chemical exposure (Onile *et al.*, 2016; Der *et al.*, 2015; Khaled, 2013; Elsebai, 1977).

Whereas females had higher prevalence of the pathologies than males in the archived samples, in the community samples**,** males had a higher prevalence of bladder pathologies than females Analyses also showed that females were more likely to develop bladder pathologies than males. Males were 41% less likely to develop bladder pathologies than females but this is unexplaned and needs to be investigated further using genomic study. Again, the variance could be that patients' biopsy samples were from sick patients with symptoms who had reported to the hospital for laboratory diagnosis whilst urine samples were from asymptomatic population from the communities. With regard to the community samples, the analysis showed that participants aged between 6-19 years were 1.76 times more likely to have bladder pathologies with reference to those above 60. This could be due to the fact that the younger aged group does not have stronger immunity against *S. haematobium* infection as compared to older age group.

Cytological, histological and experimental studies have strongly associated urinary schistosomiasis with bladder pathologies in endemic communities (Suillivan *et al.*, 2010). Epidemiological studies

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had revealed that in communities where *S. haematobium* infection is endemic, bladder pathology is prevalent (Lotan *et al.*, 2012). There was geographical correlation between urinary schistomiasis and bladder pathologies in the community participants, but this does not necessarily guarantee causality. There was, however, 6.8 times likelihood of someone with urinary schistosomiasis developing bladder pathology than someone without it. The epithelial bladder pathologies in association with urinary schistosomiasis observed in this study were squamous metaplasia, hyperkeratosis, transitional cell carcinoma, squamous cell carcinoma and urothelial dysplasia but there was a significant association between squamous cell carcinoma and urinary schistosomiasis in archived samples. Patients who were diagnosed with squamous cell carcinoma were 6.8 times more likely to test positive for *S. haematobium* infection as compared to those patients who tested negative for the disease. Patients who were diagnosed with transitional cell carcinoma were 64% less like to test positive for schistosomiasis as compared to those who tested negative for the disease. Also, patients who were diagnosed with chronic cystitis were 55% less likely to test positive for schistosomiasis as compared to those who tested negative for chronic cystitis. Intensity of infection could dictate disease progression. Few worms lay few eggs, many worms lay many eggs hence high intensity of infection. Intensity of infection might have influenced progression of the bladder pathology. These findings were similar to those of studies in Egypt and South Korea where pre-neoplastic lesions like SM, UD and hyperkeratosis in people with urinary schistosomiasis was observed in archived biopsy samples with ova count increasing with progression of the disease (Chala *et al.*, 2017; Botelho, Oliveira, Lopes, Correia da Costa, & Machado, 2011). Similar studies observed the prevalence of SCC was about 36% associated with urinary schistosomiasis (Kyritsi, *et al.*, 2018), whereas 65% prevalence rate of SCC has been observed in urinary schistosomiasis endemic community in Nigeria (Mungadi *et al.*, 2008). A retrospective histopathological study in Tanzania revealed SCC is associated with *S.*

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haematobium infection (Rambau, Chalya, & Jackson, 2013). A similar study by Bhagwandeen (1976) in Zambia found association between urinary schistosomiasis and bladder pathologies occurring in younger people in endemic communities. The study found out that patient with TCC and chronic cystitis were less likely to test positive for urinary schistosomiasis.

The mean CE concentration of controls was significantly lower than those of all the pathologies. High significant differences were observed in mean concentration of CE among the bladder pathologies with the differences increasing with severity of bladder pathologies and mean CE concentration. The mean concentration of CE of UD and HK were also significantly higher than that of SM. Bladder pathologies caused by urinary schistosomiasis had been associated with catechol oestrogen (CE). The mean concentration of CE in urine was different for the different categories of bladder pathologies, with the concentration increasing with the severity of the disease. This could imply that high CE levels contributed to severity of the disease.

The mean egg counts of controls were significantly lower than those of all the pathology groups. The difference in mean ova count between controls and all pathology groups was significant because the controls were supposedly negative for *S. haematobium* infection hence no presence of ova. The differences in *S. haematobium* mean ova count and mean CE concentration among categories of bladder pathologies indicate that the ova counts and CE concentration for various categories of bladder pathologies were different. The mean ova counts and CE concentration seemed to increase with severity of the bladder pathology. Comparisons between pathologies also showed highly significant variations for all pairs with UD having the highest counts and HK the least

S. haematobium ova counts against concentration of CE revealed a highly significant correlation between ova counts and CE concentration in urine. The mean ova count and mean CE concentration

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was least in hyperkeratosis and highest in urothelial dysplasia (precancer lesion) in community samples. The correlation was stronger in people with severe infection. Since CE is high in high ova count, it implies high rate of metabolism to yield semiquinone was high hence high genotoxicity. High genotoxicity may cause more damage to DNA. This could indicate that hormonal factor could influence disease progression. The differences in concentration of catechol oestrogen in the different categories of bladder pathologies could imply that varying levels of CE was associated with progression of bladder pathologies. Pairwise comparison showed much difference in mean concentration between control (false positive) and any of the bladder pathologies (true positive) suggesting it has the potential to be used as biomarker

A study by Mostapha *et al.* (1999) found different concentration of CE in different bladder pathologies. The higher the level of CE the higher the degree of pathology and vice versa but this does not imply causality.

The participants in the *S. haematobium* endemic community with the infection were treated with praziquantel. When comparison of mean catechol oestrogen levels in pretreatment urine was compared with post treatment mean CE levels in same participants, it was observed that, there was exponential reduction in CE levels from week 1 to 4 post treatment.

Some smears showed various stages of bladder pathologies in different fields of the same smear or different urine smears from the same study participants. This observation is due to the fact that all pathologies and in this case, bladder pathology is a progressive disease and at any point in time the various stages of bladder pathologies shed cells from the bladder epithelium into urine sample.

Cystoscopy and other molecular markers requiring sophistication and invasive sampling procedures has been the screening tool for bladder pathologies. In this study, urine was used and there was a

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positive correlation between CE concentration and urinary schistosomiasis, CE and *S. haematobium* ova count,, CE and bladder pathologies, Therefore to find out if CE could be used as a biomarker for diagnosing bladder pathologies in urine, Receiver Operating Characteristic (ROC) curves was used and the corresponding area under the curves (AUCs) show that CE has an excellent predictive ability to discriminate bladder pathology patients from normal individuals. Therefore, CE can be used as biomarker in screening for people at risk of developing or have developed bladder pathologies in communities where risk factors are prevalent.

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CHAPTER SIX

6.0 CONCLUSION AND RECOMMENDATION

6.1. Conclusion

This study indicates an overall prevalence of urinary schistosomiasis of 19.2% in patients archived biopsy samples and 9.7% at Zenu and Weija and their environs. Prevalence of *S. haematobium* infection generally varied among the age groups of the patients archived biopsy samples. Prevalence of *S. haematobium* infection was high in females in archived biopsy samples and high in males in community urine sample. Colour of urine was a strong predictor of testing positive for urinary schistosomiasis (**$p<0.05$**). There was a significant association between squamous cell carcinoma, Transitional cell carcinoma, and *Schistosoma haematobium* infection. Patients who were diagnosed with squamous cell carcinoma were 6.8 times more likely to test positive for *S. haematobium* infection as compared to those patients who tested negative for the disease.

Younger age group in the community participants were more likely to develop bladder pathologies whilst in patients (using archived biopsy sample) it was the elderly. Females were more likely to develop bladder pathology in both hospital patients and community participants. There was variation in concentration of CE in categories of bladder pathology with the level increasing severity of bladder pathologies.

The sensitivity of some biomarkers associated with bladder pathology analyzed compared with cytology, revealed that CE was most sensitive in detecting exposure to risk of developing bladder pathology or having bladder pathology.

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6.2. Recommendations

Currently diagnosis of epithelial and subepithelial bladder pathologies due to urinary schistosomiasis is based on histopathology technique using invasive procedure for sampling. The invasive method of sampling makes histopathology inappropriate as a screening tool. Urine cytology uses non-invasive sampling but has limitations as a screening tool because subepithelial bladder pathologies cannot be diagnosed. In this study CE concentration in voided urine was associated with urinary schistosomiasis, bladder pathology and *S. haematobium* ova count. Again, cut-off point (0.20 ng/ml) and above of CE concentration had been established to identify people at risk for developing bladder pathologies or having bladder pathology in *S. haematobium* endemic areas to be used as screening tool. Test kits should be developed for screening based on this.

It is recommended that further work be done on catechol oestrogen and Ki67 to evaluate the possibility of using them as diagnostic markers in rapid diagnostic test kits in addition to other test for *S. haematobium* infection to identify those who may be at risk of developing bladder pathology. This should include, standardization of urine collection (clean catch, midstream or terminal) for CE and Ki67 estimations to minimize variation. Again, lots of studies must be done to validate CE and Ki67 as biomarkers on large scale studies in combination with other biomarkers to help establish diagnostic accuracy. There must be quality control of CE and Ki67 estimations to enhance reproducibility as well as regulatory approval of the use of CE and Ki67 in clinical tests to detect early stage of bladder pathologies.

Public health education on knowledge on prevention and transmission should be intensified. Government should provide skill training for people with occupational exposure to schistosomiasis so they can change jobs. Alternative recreation facility and pipe borne water should be provided to

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curb the use of contaminated water for swimming and household chores respectively. Safe pipe borne water should be accessible in homes for domestic and commercial use to reduce contact with contaminated water. One Health approach should be used to eliminate the disease.

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APPENDICES

Appendix 1: Ethical clearance

NOGUCHI MEMORIAL INSTITUTE FOR MEDICAL RESEARCH
Established 1979A Constituent of the College of Health Sciences
University of Ghana

INSTITUTIONAL REVIEW BOARD

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My Ref No: DF22
Your Ref. No:

6th November, 2019

ETHICAL CLEARANCE

FEDERALWIDE ASSURANCE FWA 00001824 **IRB 00001276**

NMIMR-IRB CPN 038/18-19 ***revd. 2019*** **IORG 0000908**

On 6th November 2019, the Noguchi Memorial Institute for Medical Research (NMIMR) Institutional Review Board (IRB) at a full board meeting conducted continuing review and renewed your protocol titled:

TITLE OF PROTOCOL : **Urinary schistosomiasis and the state of the bladder: The role of catechol oestrogen**

PRINCIPAL INVESTIGATOR : **Ama Afrah, PhD Cand.**

Please note that a final review report must be submitted to the Board at the completion of the study. Your research records may be audited at any time during or after the implementation.

Any modification of this research project must be submitted to the IRB for review and approval prior to implementation.

Please report all serious adverse events related to this study to NMIMR-IRB within seven days verbally and fourteen days in writing.

This certificate is valid till 5th November, 2020. You are to submit annual reports for continuing review.

Signature of Chair: .....
Mrs. Chris Dadzie
(NMIMR – IRB CHAIR)

INTEGRI PROCEDAMUS

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Appendix 2: Child assent

CHILD ASSENT

	UNIVESITY OF GHANA SCHOOL OF BASIC AND APPLIED SCIENCES	

PARTICIPANT INFORMATION LEAFLET AND ASSENT FORM

TITLE OF THE RESEARCH PROJECT: THE RELATION OF CATECHOL OESTROGEN IN BLADDER PATHOLOGY OF PEOPLE INFESTED WITH URINARY SCHISTOSOMIASIS

RESEARCHERS NAME(S): Ama Afrah

ADDRESS: P. O. Box OD 29 Odorkor, Accra

CONTACT NUMBER: 0261728480

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What is RESEARCH?

Research is something we do to find new knowledge about the way things (and people) work. We use research projects or studies to help us find out more about disease or illness. Research also helps us to find better ways of helping, or treating children who are sick.

What is this research project all about?

This research is about a disease called Bilharziasis, have you ever heard of it before? It is caused by a group of small animals called *Shistosoma* that leave in water or river. When one goes swimming, fishing or have any contact with water that contain this Schistosoma, they penetrate the skin and find their way into some of the organs. There are various types of the *Shistosoma* so they affect various parts of the body depending on type. My research is about the type called *Schistosoma haematobium* which affects the bladder, the organ in our body where urine is stored. *Schistosoma haematobium* in the bladder cause a lot of disturbances which affects the health of the cells in the bladder resulting in blood in urine. Some people have the disease, but they do not know about it and when not checked and treated can result in bladder cancer in the future. This research wants to find those which have the disease and tell the government which includes the president of Ghana to help such people especially you children because you are our future leaders and I and my team wants you to be healthy for the future role.

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Why have I been invited to take part in this research project?

You have been invited to take part in this research so that we know whether you have a healthy bladder or not. If you do not have a healthy bladder, I and my team with arrange to get you treated so you will be healthy now and the near future.

Who is doing the research?

I am Ama Afrah from the University of Ghana and I want to know how many people have the disease (Bilharzia) that is likely to develop bladder cancer if not treated early.

What will happen to me in this study?

I will give you a container so that you give me about 10 table spoons full urine. I will test first for the presence of blood and later cell from the bladder in the urine stained and examined under the microscope to see what is going on in the bladder due to the disease.

Can anything bad happen to me?

This will not cause any pain, it's just like going to the washroom to pee.

Can anything good happen to me?

The good thing is that if you have the disease, it will be identified and treated. The river or water that is infected will be treated so that in future anyone that gets into contact with it will not get sick.

Will anyone know I am in the study?

I will not tell anybody about you been part of this research but the results is what will be used to get treatment for you and other children who may also have the disease even in the future.

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Who can I talk to about the study?

I am leaving my phone number and that of my supervisors so that if you have any questions feel free to ask.

Name	Phone number
Ama Afrah	0261728480
Dr. B. Kwansa-Bentum	0244858495
Dr.Fred Futagbi	0244993383
Prof. Patience B. Tetteh-Quarcoo	0244633251
Prof. R.K. Gyasi	0233632427

What if I do not want to do this?

I have to ask permission from your parents or guardian but even if they agree to do it but you do not want to take part, you are free to do so. Nothing will happen to you.

Do you understand this research study and are you willing to take part in it?

YES	NO

Has the researcher answered all your questions?

YES	NO

Do you understand that you can pull out of the study at any time?

YES	NO

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_____ _____

Signature of Child Date

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Appendix 3: Participation leaflet and informed consent form

PARTICIPANT INFORMATION LEAFLET AND CONSENT FORM

TITLE OF THE RESEARCH PROJECT: The Relationship Between Catechol Oestrogen And Bladder Pathologies In Human Urinary Schistosomiasis

REFERENCE NUMBER: # 038/18-19

PRINCIPAL INVESTIGATOR: Ama Afrah

ADDRESS: P.O.Box OD 29 Odorkor, Accra.

CONTACT NUMBER: 0261728480

You are being invited to take part in a research project. Please take some time to read the information presented here, which will explain the details of this project. Please ask the study staff any questions about any part of this project that you do not fully understand. It is very important that you are fully satisfied that you clearly understand what this research entails and how you could be involved. Also, your participation is not compulsory and you are free to refuse to participate.

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If you say no, this will not affect you negatively in any way whatsoever. You are also free to withdraw from the study at any point, even if you do agree to take part.

This study has been approved by the Institutional Review Board (IRB) of Noguchi Memorial Institute of the University of Ghana and will be conducted according to the ethical guidelines and principles of the IRB that conform to international standards.

What is this research study all about?

The research will be conducted at Zenu, and Weija and their environs all in Accra, Ghana. Three hundred and eighty-six (386) people will take part in the study.

This research is about a disease called Bilharziasis, have you ever heard of it before? It is caused by a group of small animals called *Shistosoma* that leave in water or river. When one goes swimming, fishing or have any contact with water that contain this Schistosoma, they penetrate the skin and find their way into some of the organs. There are various types of the *Shistosoma* so they affect various parts of the body depending on type. My research is about the type called *Schistosoma haematobium* which affects the bladder, the organ in our body where urine is stored. *Schistosoma haematobium* in the bladder cause a lot of disturbances which affects the health of the cells in the bladder resulting in blood in urine. Some people have the disease, but they do not know about it and when not checked and treated can result in bladder cancer in the future. This research wants to find those which have the disease and tell the government which includes the

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president of Ghana to help such people especially you children because you are our future leaders and I and my team wants you to be healthy for the future role.

Two clean containers will be given to you to provide urine. Your urine will be put in a small container and cells separated using a machine called centrifuge. Part of the cells will be put on 2 slides and stained and examined under the microscope. The second container I am going to measure a substance called catechol oestrogen which may be from the Schistosoma egg, the microorganism that cause the disease.

After the research the people who agreed to take part and may have the disease will be given medication.

Why have you been invited to participate?

You have been invited to take part in this research to know if this disease affects children like you because if not treated, it may affect your future.

What will your responsibilities be?

The part you will play that is if you agree to be part of this study is to sign this paper and give me your urine.

Will you benefit from taking part in this research?

This research is going to help the government of Ghana to know how many people (men, women, children and adults) in your area and the others I mentioned to you have the disease, to know if how old one is influence having the disease, to know if being a female or male have any influence on the disease, if catechol oestrogen in the Schistosoma ova can cause some changes in the bladder cells.

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You may not benefit directly from this project but the findings will help government take good decision on other children like you in the future so they do not get this very disease.

Are there in risks involved in your taking part in this research?

Taking part in this research will not harm you.

If you do not agree to take part, what alternatives do you have?

You can decide not to take part, but this will not affect you in anyway. Even if you agree to take part but decide not to continue, I will gladly allow you to redraw.

In case you have the disease, your parents or guardian can go to the nearest clinic or hospital for treatment.

Who will have access to your medical records?

Nobody is going to have access to your report except myself, my supervisors and examiners and a few publishers of the findings of this research without mentioning your name.

Will you be paid to take part in this study and are there any costs involved?

No you will not be paid to take part in the study. There will be no costs involved for you, if you do take part.

Is there anything else that you should know or do?

You can contact the IRB at 021-938 9207 if you have any concerns or complaints that have not been adequately addressed by your principal investigator.

You will receive a copy of this information and consent form for your own records.

Declaration by participant

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By signing below, I ……..……. agree to take part in a research study entitled 'The Relation of Catechol Oestrogen In Bladder Pathology Of People Infested With Urinary Schistosomiasis'

I declare that:

I have read or had read to me this information and consent form and it is written in a language with which I am fluent and comfortable.

I have had a chance to ask questions and all my questions have been adequately answered.

I understand that taking part in this study is voluntary and I have not been pressurized to take part.

I may choose to leave the study at any time and will not be penalized or prejudiced in any way.

I may be asked to leave the study before it has finished, if the study doctor or researcher feels it is in my best interests, or if I do not follow the study plan, as agreed to.

Signed at (*place*) .....…..…….. on (*date*) ……...…….. 2022.

Signature of participant **Signature of witness**

Declaration by investigator

I *(name)* ……..…… declare that:

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I explained the information in this document to ……

I encouraged him/her to ask questions and took adequate time to answer them.

I am satisfied that he/she adequately understands all aspects of the research, as discussed above

I did/did not use a interpreter. (*If a interpreter is used then the interpreter must sign the declaration below*.

Signed at (*place*) .....…..…….. on (*date*) ……...…….. 2022.

Signature of investigator Signature of witness

Declaration by interpreter

I *(name)* ……..…… declare that:

I assisted the investigator (*name*) ……. to explain the information in this document to (*name of participant*) ……..…….. using the language medium of ……

We encouraged him/her to ask questions and took adequate time to answer them.

I conveyed a factually correct version of what was related to me.

I am satisfied that the participant fully understands the content of this informed consent document and has had all his/her question satisfactorily answered.

Signed at (*place*) .....…..…….. on (*date*) ……...……..

Signature of interpreter Signature of witness

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INFORMED CONSENT FORM

Title: The Relation of Catechol Oestrogen In Bladder Pathology Of People Infested With Urinary Schistosomiasis

Investigator

Ama Afrah, BSc., M.Phil.

PhD Candidate; Dept. of Animal Biology and Conservation Science, University. of Ghana; Legon

Tel: +233 261728480

E-mail: aafrah@st.ug.edu.ghmailto:gfutagbi@ug.edu.gh

Or: amafrh@yahoo.co.ukmailto:fgodfred@yahoo.com

What is RESEARCH?

Research is something we do to find new knowledge about the way things (and people) work. We use research projects or studies to help us find out more about disease or illness. Research also helps us to find better ways of helping, or treating children who are sick.

Introduction

We kindly request you take part in study/research which I will proceed to describe to you. We would like to start by saying that this study/research is strictly voluntary. Should you, at any point during the study/research, decide that you do not wish to participate any further, you are free to terminate the participation of yourself immediately. Any such decision will be respected without any discussion and will not adversely affect you in anyway. The study involves schistosomiasis/Bilharzia and its effect on the health of your bladder.

In order to be sure that you are informed about being in this research, we are asking you to read this Consent (consent is an agreement) Form or it will be read to you if you cannot read. We also

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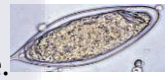
require that you sign this Consent Form in front of a witness. We will give you a copy of this form. This consent form might contain some words that are unfamiliar to you. Please ask us to explain anything you may not understan

Reason for the Research

You are being asked to take part in research to help us find a more insight to the damage *Schistosoma haematobium,* the parasite that cause Bilharzia, that is blood in urine. This research is about a disease called Bilharziasis, have you ever heard of it before? It is having blood in one's urine. It is caused by a group of small animals called *Shistosoma* that leave in water or river not sea. When one goes swimming, fishing or have any contact with water that contain this Schistosoma, they pass through the skin and find their way into some of the important organs like bladder. There are various types of the *Shistosoma* so they affect different parts of the body depending on type. They can affect lung, bladder and intestine. My research is about the type called *Schistosoma haematobium* which affects the bladder. Bladder the organ in our body where urine is stored.

This is a picture of *Schistosoma haematobium* egg with a spine.  . *Schistosoma haematobium* when in the bladder uses its spine to chuck the bladder resulting in sore and eventually blood appears in urine. The egg of the *Schistosoma haematobium* also contains a fluid called catechol oestrogen a substance which can change shape and normal function of the bladder and if not checked, cause cancer of the bladder. Some people who live close to some rivers and streams which contains *Schistosoma haematobium* like your community have the disease but they

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do not know about it and when not checked and treated can result in bladder cancer and eventual death in the future.

The study involves adults and children with blood in their urine who live close to streams or rivers that have this *Schistosoma haematobium*. The study may also help to understand how the bladder become abnormal in structure and function which affect your bladder and your health in general.

General Information about Research

Currently poor sanitation meaning people with the disease urinating and defecating into rivers/streams/environment, lack of potable water that is not having clean drinking water, illegal mineral extraction that is galamsey and future irrigation dams' construction that is watering of farm produce by digging wells are major problems in *Shistosomiasis* infection control. Ghana has a sanitation coverage of 15% failing to meet MDG subsection 7 where we were tasked to get sanitation coverage of about 70%, meaning the environment of Ghana as a whole is not clean at all. The prevalence rate meaning how many people having *Schistosoma haematobium* in Ghana is not up to date therefore bladder cancer due to *Schistosoma haematobium* infection is also not known but a survey (a little) of school children in some selected communities diagnosed routinely of *Schistosoma haematobium* infection also have a 16.2% rate prevalence of urothelial dysplasia, a pre-cancer lesion, chronic inflammation. Which means that in a certain community with *Schistosoma haematobium* infection, for every 100 children, more than 16 children have problems with their bladder which was seen when they gave their urine out to be checked for the state of their bladder due to the infection. This must be looked into further and appropriate control measures taken and treatment given to those infected as well as their streams/rivers also gotten rid

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of *Schistosoma haematobium* by the government to address the situation in communities where the disease is.

In addition, schistosomiasis affect we in the developing countries like Ghana because the effect of the disease results in children dropping out of school as well as adults also not been effective at work. Such people cannot work, save and countribute to development of Ghana.

Recurrent *Schistosoma haematobium* infections when not treated can cause a lot of health problems including bladder cancer which can later spread to other parts of the body and eventually cause death. The incidence of *Schistosoma haematobium* in Ghana keep increasing due to increased exposure to risk factors and lack of sustainable control program. Partaking in this study will help you know the state of your bladder to help government bring lasting solution to the problem for better health and development.

All information collected from you will be kept secret. Your results will be put on paper and handed over to you personally with explanation and kept secret as well.

There will be laboratory tests on urine to see if you have the *Shistosoma* parasite and the effect the disease have on your bladder cells due to a substance (catechol oestrogen) the parasite contains in its ova. The amount of urine will not exceed 10 tablespoonsful and will not harm you.

Your Part in the Research

If you agree to be in the research, you will be asked to allow for the collection of urine samples as soon as possible. Samples will be collected only once. So your part in the research ends once the samples are collected.

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Possible Risks

There is no risk associated with providing urine sample.

Possible Benefits

There are no immediate, direct benefits to you. The results of the tests to be carried out will, however, be made available to you. Depending on what is found, the information may or may not provide assistance to you. It is hoped that in the future the information from the study may help in the better diagnosis, management, control, and prevention of urinary schistosomiasis in Ghana.

Confidentiality

We will protect information about you taking part in this research to the best of our ability. You will not be named in any reports. However, the examiners, supervisors and some papers may sometimes look at your research records. Someone from the Institutional Review Board (IRB), those who gave me permission to do this research might want to ask you questions about being in the research, but you do not have to answer them. A court of law could order medical records shown to other people, but that is unlikely.

Compensation

There will be no financial burden placed on you if you should agree to participate.

If You Have a Problem or Have Other Questions

Please call if you have any questions relating to this consent form or the study itself, please contact the above principal investigator.

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Your rights as a participant

This research has been reviewed and approved by the University of Ghana, Institutional Review Board of Noguchi Memorial Institute for Medical Research. If you have any questions about your rights as a research participant you may contact the principal investigator, Ama Afrah, on Tel: 0 2261728480.

Thank you for your time and participation.

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Appendix 4: Consent Form for Parent/Guardian

PARENTAL/GUARDIAN CONSENT FORM

TITLE OF STUDY: The Relation of Catechol Oestrogen In Bladder Pathology Of People Infested With Urinary Schistosomiasis

I_____ [insert full name] is the legal parent or guardian of [insert full name]:_____ and I agree that my minor child named should take part in the study relating to what will happen to the part of the body that store urine if it my child is infected with a germ that live in river or stream when you come into with it as happens in my community. I also agree that you put findings in a paper for people to read or to be used for the purpose of presenting it to the public.

I understand the following:

The Information will be put in a paper without my child's name/ward name attached and every attempt will be made to ensure nobody sees the name. I understand, however, that complete namelessness cannot be guaranteed. It is possible that somebody somewhere - perhaps, for example, somebody who looked after my child/ward if I was in hospital, or a relative - may identify me.

The Information may be published in a paper which is read in the whole world or a paper on the internet. Papers are those that are concerned with the health of people but may be seen by many who are not in the health field including journalists.

I can withdraw my agreement at any time before the results is put on the internet, but once the Information has been put in a paper or the internet, it will not be possible to withdraw the agreement.

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Signature of Parent/Guardian: _____ Date: _____

Signature of requesting principal investigator: _____Date:_____

What is this research study all about?

The research will be conducted at Zaenu, Ada and Weija all in Greater Accra Region of Ghana. The reason for choosing these places is that they all have rivers/streams that have germs in them. Children are the most affected.

Your child/ward is being asked to take part in study to help us find a more understanding of the damage the germ cause to the parts of the body that stores urine. Children can get this germ when they go swimming, fishing, and farming or cross the river or stream to school or any other place and the water that contain the germs touches the skin. The germs pass through the skin first, then moves to the blood and finally gets to the part of your body that stores urine. The germ has a tail that looks like a pin. The germ also contains something called catechol oestrogen that can

The germ uses its tail to prick the part of the body that stores urine resulting in sore. You know sore bleeds and finally blood appears in urine. The germ can change shape of the part of the body that stores urine and this can result in one not been able to store urine, not able to urinate and finally develop a disease call cancer. Cancer is very difficult and expensive to treat.

The disease cancer can kill. The disease cancer can spread to other parts of the body when not treated early. Some people who live close to rivers and streams which contains the germ like your community have the disease but they do not know about it and when not checked and treated can result in health complications and eventual death in the future.

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More people keep getting the disease but the how many people having it is not known so the government cannot help such people.

Although the exact number of people are not known, a few number of school children in some selected communities where the river contains the germ, more than 16 children out of every 100 children are showing signs of the disease cancer. This is a serious problem especially because there are children. This must be looked into further and appropriate control measures taken and treatment given to those infected as well as their streams/rivers also gotten rid of the germ by the government to address the situation in communities where the disease is found.

Children drop out of school because the disease affects children's ability to learn.

Allowing your child/ward to partak in this study will help you know the state of the part of your child's/ward's body where urine is stored because he/she lives close to river/stream that contain the germ. The results we get from this study will help government bring lasting solution to the problem for better health and development of all children.

There will be laboratory tests on urine to see if your child/ward have the germ and the effect the germ have on the part of your child/ward's body where urine is stored. The amount of urine will not exceed 10 tablespoonsful and will not harm your child/ward.

What is required of your child/ward/relative?

Your child/ward will be given clean container to provide urine. His/her urine will be put in a small container and small living things called cells from the part of the body where urine is stored will be separated from the fluid. Part of the cells will be put on 2 glass slides and stained using colours and coloured cells looked at under the microscope. What the microscope does is that, very

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small things that the eyes cannot see, the microscope will made it appear very big like 40 times or 100 times bigger. For the second container of urine, I and my team are going to measure a substance called catechol oestrogen which will be in the urine due to the presence of Schistosoma haematobium egg in the bladder, which may cause bladder cancer.

After the research the people who agreed to take part and may have the disease may be given medication by Centre for Disease Control (CDC).

A total of one hundred and ninety -four people (children and adults) from the communities mentioned will take part in the study.

Why have you been invited to participate?

You have been invited to agree for your child/ward/relative to take part in this research to know if this disease affects child because if not treated, it may affect his/her future.

What will your responsibilities be?

The part you will play that is if you agree for your child to be part of this study, is to sign this paper and allow him/her to give me his/her urine.

Will you benefit from taking part in this research?

This research is going to help the government of Ghana to know how many children in your community and the others I mentioned to you have the disease, to know if how long one is exposed to germ effect having the disease, to know if being a female or male have any effect on the disease, if catechol oestrogen in the germ can cause some changes in the bladder cells and eventually bladder cancer.

Your child/ward may not benefit directly from this project but the findings will help government take good decision on other children in the future so they do not get this very disease.

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Are there in risks involved in your ward taking part in this research?

Taking part in this research will cause very very little or not harm your child or ward because I am not touching him/her with anything.

If you do not agree for your child/ward to take part, what alternatives do you have?

You can decide not to allow your child/ward to take part but this will not affect your child/ward in anyway. Even if you agree for your child to take part but decide not to continue, I will gladly allow your child/ward to redraw.

In case your child/ward have the disease you can take your child/ward to the nearest clinic or hospital for treatment.

Who will have access to your medical records?

Nobody is going to have access to your child/ward's report except myself, my supervisors and examiners and a few writers of the findings of this study without mentioning your child/ward's name.

Privacy

Any information been it age, gender, how long you have been living in your community, any knowledge on the germ will be collected and kept secret.

Will you be paid to take part in this study and are there any costs involved?

No you will not be paid to take part in the study. There will be no costs involved for you, if your child/ward do take part.

Is there anything else that you should know or do?

You can contact the Institutional Review Board (IRB) of Noguchi Memorial Institute for Medical Research (NMIMR) of the University of Ghana at +233-302501178/+233-302501179 if you have any concerns or complaints that have not been adequately addressed by the principal investigator.

You will receive a copy of the consent form for your own records.

Declaration by Parent/Guardian

By signing below, I ……..……. agree to allow my child/ward to take part in a study entitled 'Urinary Schistosomiasis and The State of The Bladder: The Role of Catechol Oestrogen'.

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I declare that:

I have read or had read to me this information and consent form and it is written in a language with which I am fluent and comfortable.

I have had a chance to ask questions and all my questions have been adequately answered.

I understand that my child/ward/relative taking part in this study is voluntary and I have not been pressurised to allow my child/ward to take part.

I may choose to let my child/ward leave the study at any time and will not be penalised or discriminated upon in any way.

I may be asked to allow my child/ward/relative to leave the study before it has finished, if the researcher feels it is in my best interests, or if I do not follow the study plan, as agreed to.

Signed at (place) .....…..…….. on (date) ……...…….. 2019.

Signature of parent/guardian.....…..…. Signature of witness

Declaration by investigator

I (name) ……..…… declare that:

I explained the information in this document to ……..

I encouraged him/her to ask questions and took adequate time to answer them.

I am satisfied that he/she adequately understands all aspects of the research, as discussed above

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I did/did not use a interpreter. (If an interpreter is used then the interpreter must sign the declaration below.

Signed at (place) .....…..…….. on (date) ……...…….. 2019.

Signature of investigator……..Signature of witness……

Declaration by interpreter

I (name) ……..…… declare that:

I assisted the investigator (name) ……. to explain the information in this document to (name of participant) ……..…….. using the language medium of ……

We encouraged him/her to ask questions and took adequate time to answer them.

I conveyed a factually correct version of what was related to me.

I am satisfied that the participant fully understands the content of this informed consent document and has had all his/her question satisfactorily answered.

Signed at (place) .....…..…….. on (date) ……...……..

Signature of interpreter……..Signature of witness……..

If You Decide Not to Be in the Research

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You are free to decide if you want to be in this research. Your decision will not affect you in anyway.

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Appendix 5: Questionnaire

QUESTIONNAIRE

Title of Research: THE RELATIONSHIP BETWEEN CATECHOL OESTROGEN AND BLADDER PATHOLOGIES IN HUMAN URINARY SCHISTOSOMIASIS

Gender: F [ ] M [ ]

Location: .....

Occupation: Fisherman/fishmonger/crop farmer [ ] others [ ].

How dispose of human waste during fishing into the water: yes [ ] no [ ].

Source of Domestic Water: River/Dam [ ] Pipe Borne [ ] Both Sources [ ]

Do you bath/swim/wash with dam water: No [ ] Yes [ ] If Yes How Often in a Week: [ ] Once [ ] More Than Once [ ]

Presently do you experience blood in Urine……. No [ ] Yes [ ]

Any current bladder/urinary tract disease…… ……..No [ ] Yes [ ]

History of Blood in Urine (Haematuria)…… …No [ ] Yes [ ]

History of *S. haematobium* Infection/bilharzia last 5 years: …..No [ ] Yes [ ]

History of bilharzia treatment in last 5 years …..No [ ] Yes [ ]. If yes what type of: herbal [ ] orthodox/praziquantel [ ]

History of Smoking: No [ ] Yes [ ]……...For How Long ago……

History of Industrial Work: No [ ] Yes [ ] If Yes, How Long ago ……

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History of last MDA: No [ ] Yes [ ] If Yes, How Long ago ……

Level of Education: NE [ ] Pri [ ] Sec [ ] Ter [ ]

Knowledge on Schistosomiasis Transmission: No [ ] Yes [ ]. What exactly do you know about it
……
……
……
……

Knowledge on Knowledge on Schistosoma Prevention: No [ ] Yes [ ]. What exactly do you know about it
……
……
……
……

Signature ……..

Thump Print……

Thank You for Your Participation.

Code NE: no education

PRI: Primary education,

SEC: Secondary education

TER: Tertiary education

MDA: Mass Drug Administration

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Appendix 6: Preparation 95% of Ethanol for Pap staining Technique

The wet fixation (preservative) of the cells in the urine sediment, dehydration and removal of excess Orange G6 and Eosin Azzure in Pap staining technique uses 95% ethanol. In preparation of 95% ethanol, 95 parts of absolute ethanol was mixed with 5 parts of distilled water; 3 liters of 95% ethanol was prepared for the Pap staining technique. Two thousand eight hundred and fifty (2850) ml of absolute ethanol was measured with measured cylinder and topped up with 150 ml of distilled and mixed to prepare the 3000ml (3 liters) of 95% ethanol. This was put in a clean gallon, covered with the lid and mixed well by shaking the container and stored in a cool dry flame free area at room temperature.

Appendix 7: Preparation of Harris Haematoxylin stain, Orange Green (G) 6 and Eosin Azzure

The Pap stain (polychromatic) contains 3 main stains: haematoxylin, Orange Green 6 and Eosin Azzure/Alcohol.

The stains (Harris Haematoxylin stain, Orange G 6 and Eosin Azzure) were commercially prepared. The recommended haematoxylin for cytology in general and hence urine cytology for the purpose of this study was Harris haematoxylin because it stains the cells progressively so there is no need for remove excess stain by acid differentiation which may dislodge the urothelial cells for the slides source: https:www.cellpath.com.clear download 4110-barrisiffu00 951133vl.pdf(assessed 8/3/2023)

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Appendix 8: BIOMATIK Catechol-O-Methyltransferase (COMT) assay for catechol oestrogen metabolism

Standard antibody sandwich ELISA technique with greater sensitivity and accurate detection of catechol=O-methyl transferase (COMT) antigen was used. Catechol=O-methyl transferase is produced in the bodies of *S. haematobium* infected people as a result of the presence of the substrate catechol oestrogen found in the ova of *S. haematobium* to degrade the CE. In the process, an intermediate quinone with genotoxic property to DNA is formed, damaging DNA, with resultant bladder pathologies. The reaction was based on the fact that microplates provided in the kit were pre-coated with an antibodies specific to COMT. COMT antibodies bind specifically to COMT antigen in the urine sample

Reagent standard, participants' samples and controls were added to appropriate wells with a biotin conjugated antibody specific to catechol-O- methyl transferase (COMT).

Avidin conjugated to horseradish peroxide (HRP) was added to each microplate well and incubated. After TMB substrate solution was added, only those wells containing COMT, biotin-conjugated antibody and enzyme-conjugated Avidin exhibited a change in colour. The enzyme-substrate reaction was terminated by the addition of sulpheric acid solution and colour change was measured spectrophotometrically at a wavelength of 450nm. The concentration of COMT which was equivalent to concentration CE in the urine samples were determined by comparing the optical density (OD) of the samples to the standard curve.

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Appendix 9: Sample and ELISA Reagent Preparation

Prior to the preparation of the sample, all the materials needed for the ELISA were gathered and thawed to the appropriate (23 degrees Celsius) temperature, followed by reconstitution of standard, serial dilution of standard and preparation of reagent according to manufacturer's instruction.

Appendix 10: Preparation of urine sample for ELISA Technique

The participants' samples and controls were thawed to 23 ^{0} C and centrifuged at 1,000 RPM for 20 minutes as recommended by manufacturer's manual. The supernatant was pipetted into clean labelled tubes before use.

Appendix 11: Preparation of ELISA Reagent

The ELISA reagents constitute COMT standard, TMB, detection solution A, detection solution B that needed to be prepared or reconstituted according to the manufacturer's manual.

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Appendix 12: Reconstitution of standard

The test standard which accompanied the kits was reconstituted using 1.0 ml of reagent diluent. The solution of the test standard and diluent was shaken gently to avoid foaming after been kept at room temperature for 10 minutes. The concentration of the standard stock solution (COMT) was 50ng/ml. The standard stock solution was reconstituted to 10ng/ml by adding 0.5 ml of standard diluent to 500ul of standard stock solution. Seven tubes were prepared each containing 0.5ml of standard diluent to produce serial dilution of the 10ng/ml in first tube by adding 500ul of standard stock solution to 0.5ml of standard diluent. 500ul of 10ng/ml was pipetted and added to the second tube after mixing. This procedure was repeated to the 7^{th} tube here 500ml was pipetted and discarded. Each mixture was gently mixed to avoid foaming. The corresponding concentration of the serial dilutions of the standard solution was 10ng/ml, 5ng/ml. 2.5ng/ml, 1.25ng/ml, 0.625ng/ml, 0.312ng/ml, 0.156ng.ml and a tube with only standard diluent as blank with concentration of COMT been 0.0ng/ml.

Appendix 13: Preparation of Detection reagent A and B

Detection solutions A and B were thawed to 23 ^{0} C and centrifuged at 1000 RPM for a 1 minute before use. A dilution of 1 in 100 was made for the detection solutions 'A' and 'B' using their corresponding assay diluents 'A' and 'B' respectively. One of each detection solution was pipetted into a sterile labelled tube and 99 ml of corresponding diluent added and mixed gently to avoid foaming as as recommended by reagent manual.

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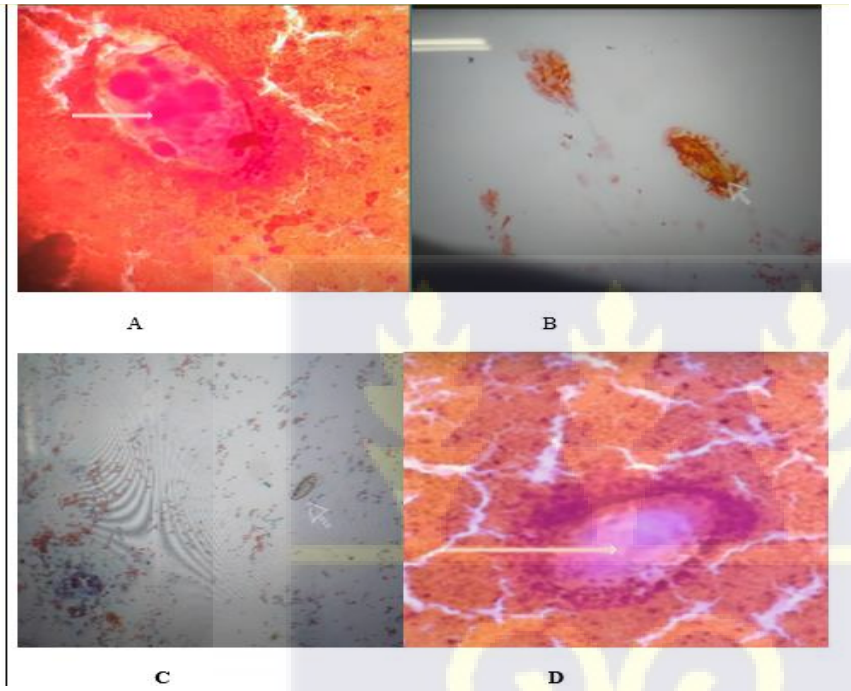
Appendix 14: Preparation of Wash solution

580ul of double distilled water was added to 20ul of wash solution concentrate to prepare 600ul of wash working solution as recommended by reagent manual.

Appendix 15: Micrograph of *Schistosoma haematobium* ova



Urine smears showing *S. haematobium* ova: (A), (B) and (C) show *S. haematobium* ova (white arrowed) and (D) is *S. haematobium* ovum trapped within chronic inflammatory cells. Pap stain. X 400

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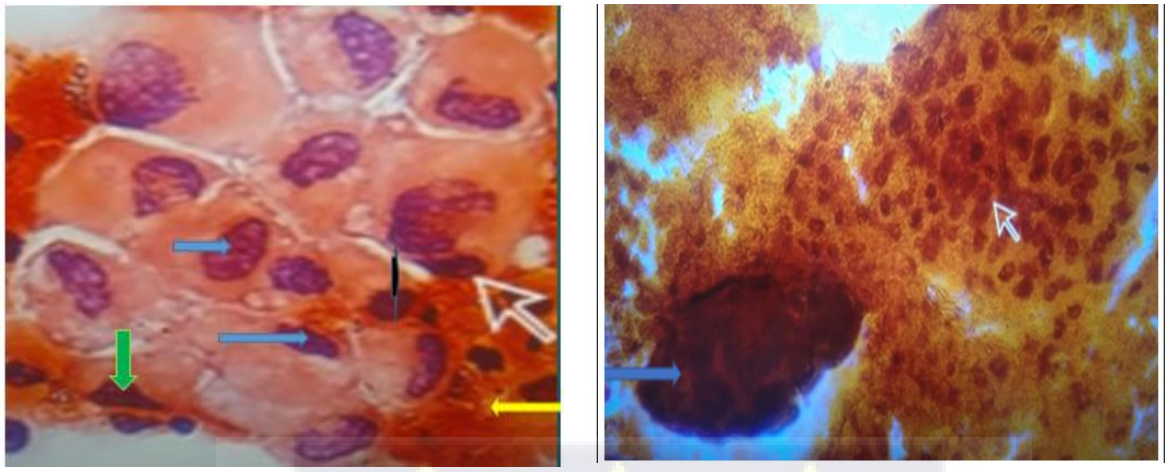
Appendix 16a: *S. haematobium* and Bladder pathologies



A1 A2

A1: Keratinizing (yellow arrow) Squamous Cell Carcinoma with pleomorphic (blue arrows) hyperchromatic (black arrow) and eccentric (green arrow) nuclei. Pap stain. X400 (x 10 magnification by eye piece X 40 magnification by objective x10).

A2: Keratinizing Urothelial Dysplasia: cluster of urothelial dysplastic cells (white arrow) with *S. haematobium* ovum (blue arrow) in Pap -stained urine smear. X 400

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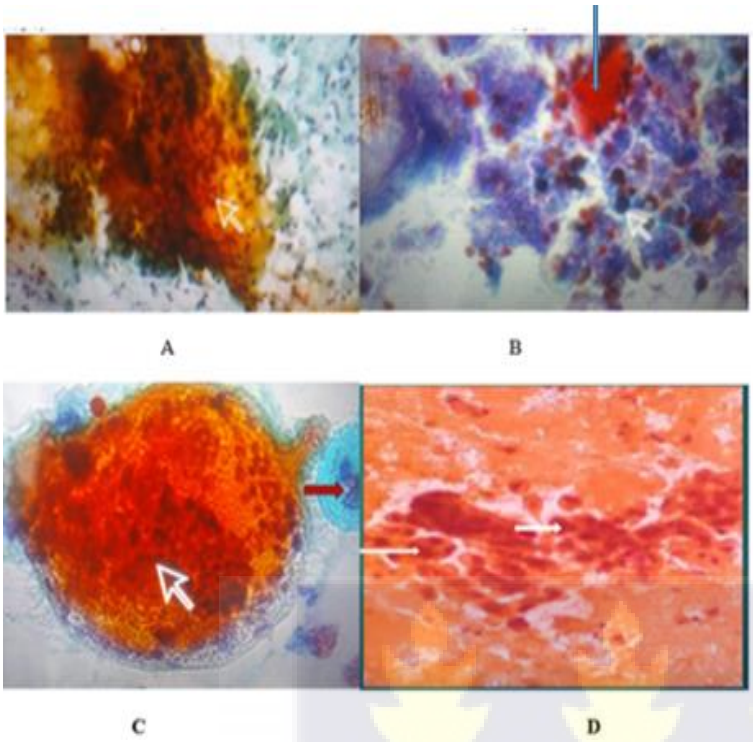
Appendix 16b: Bladder pathologies



Urine smear of urothelial dysplasia: micrograph (A): sloughed urothelium of urothelial dysplasia. Micrographs (B) show hyperchromatic keratinized cytoplasm and hyperchromatic nuclei (white arrow). Micrograph (C) show non-keratinizing (red arrow) urothelial dysplasia and what appear to be a giant cell (white arrow); (D) shows keratinizing (white arrows) urothelial dysplasia. Pap stain. X400

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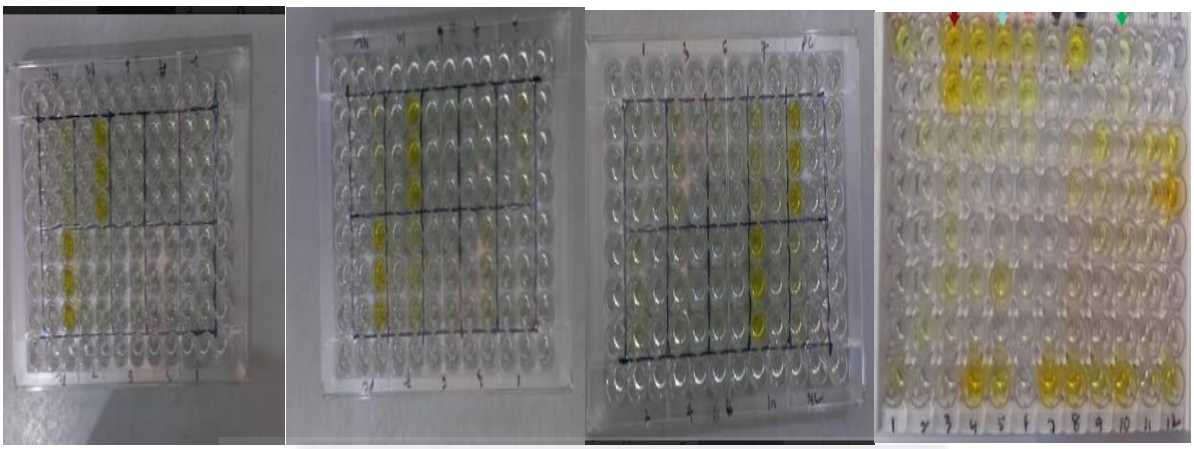
Appendix 17: ELISA Plate



ELISA plate showing varying concentrations of CE of community study participants and controls. Ting of (light, moderate, deep) yellow indicates positive catechol oestrogen

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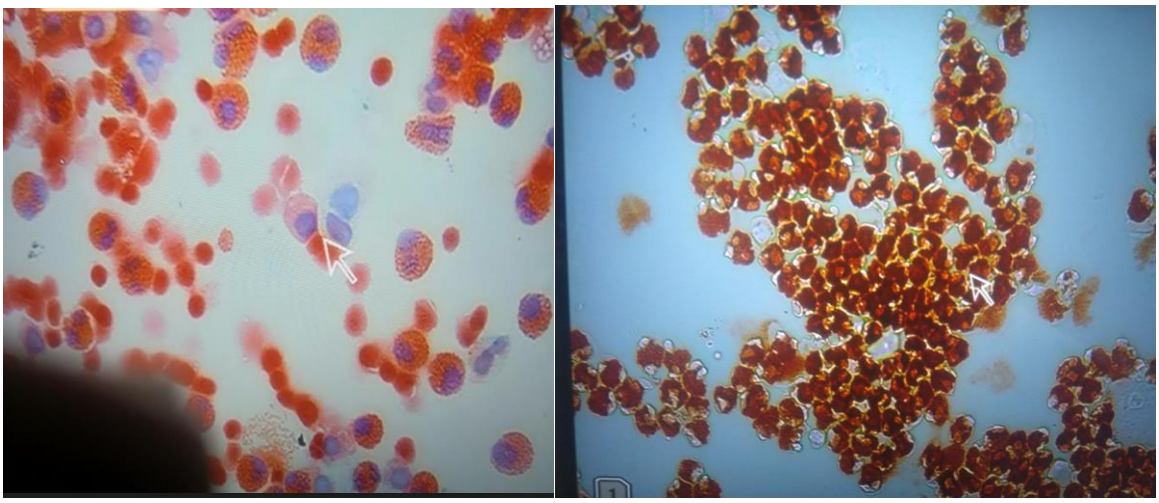
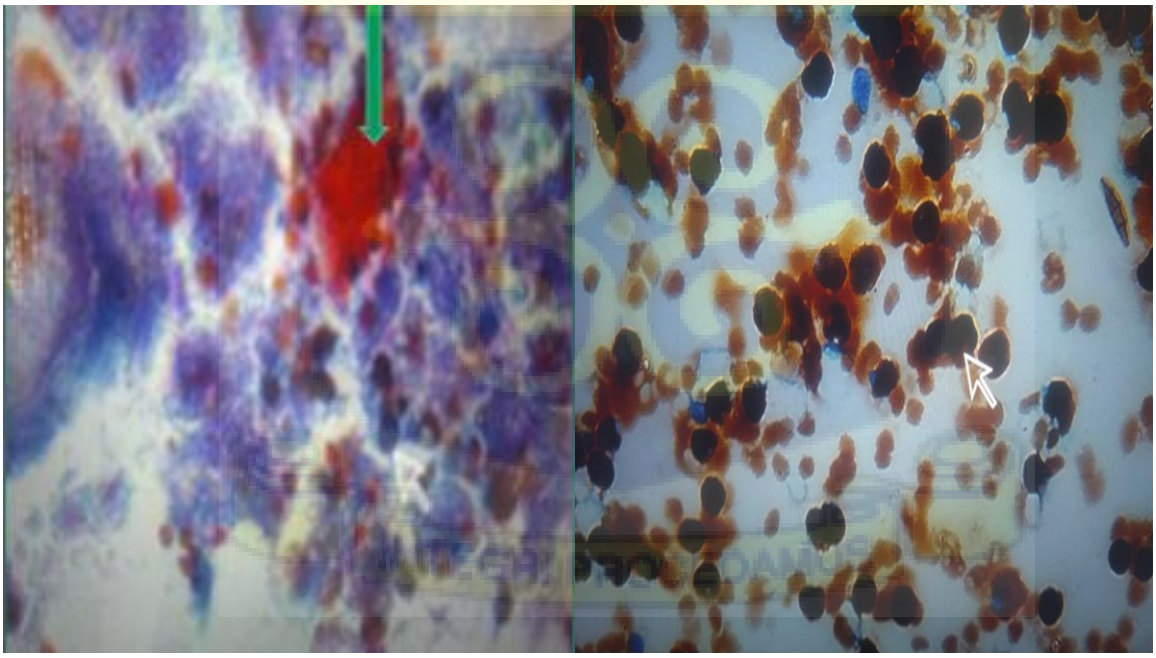
Appendix 18a: bladder pathology with corresponding Ki67 positive smear



A1 A2

Urothelial dysplasia (A1) and Ki67 positive (A2) smear white arrow).

Appendix 18 b: bladder pathology with corresponding Ki67 positive smear



A1 A2

Keratinizing urothelial dysplasia (A1) and corresponding Ki67 positive (A2) smear