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Paediatric Schistosomiasis: Diagnosis, Morbidity and Treatment

Welcome Mkululi Wami

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Abstract

Schistosomiasis is a major parasitic disease caused by parasitic helminths of the genus *Schistosoma* which affects children in Africa, with negative impacts on general health, growth and cognitive development. Infection and morbidity are controlled by treatment with the antihelminthic drug praziquantel. Preschool children (aged ≤5 years old) have been neglected both in terms of research and control, and it is only recently that the World Health Organization (WHO) recommended praziquantel treatment and the inclusion of preschool children in control programmes. However, the burden of disease in this age group still remains poorly understood, and the performance of the currently available tools for detecting infection and morbidity is still yet to be systematically evaluated. The aim of this thesis was to compare the utility of currently available tools for diagnosing *S. haematobium* infection and related morbidity. The initial study cohort consisted of 438 Zimbabwean children (age range: 1–10 years) who were endemically exposed. Point-of-care schistosome-related morbidity markers applicable in the field, as well as serological biomarkers (CHI3L1, CRP, ferritin, resistin and SLPI) and inflammatory cytokines (IL-4, IL-5, IL-10, IL-13 and IFN-γ) that could predict early stages of immune-mediated pathology due to schistosomiasis were measured. Using a combination of applied statistical methods, the effect of treatment on factors associated with *S. haematobium* exposure, infection and morbidity in children aged 1–5 years was determined and the findings compared with those observed in children aged between 6–10 years old, who are the current targets of the schistosome control programmes.

In this thesis, I able to demonstrate that preschool children carried significant infection, further reiterating the need for their inclusion in control programmes. Furthermore, this study demonstrated the importance of using additional sensitive diagnostic methods as this has implications on the required intervention strategies for the targeted populations. This study further revealed that preschool children

can be effectively screened for schistosome-related morbidity using the same currently available diagnostic tools applicable to older children. Urinalysis markers microhaematuria, proteinuria and albuminuria are recommended in this thesis as the best choice for rapid assessment of morbidity attributed to *S. haematobium* infection in the field. Additionally, it was shown that the praziquantel treatment regimens aimed at controlling schistosome infection and morbidity currently designated for primary school-aged children and older populations are applicable to preschool-aged children. The involvement of serum biomarkers and immune correlates in the biological processes of inflammation suggests that these markers can be potential early predictors of schistosome-related pathology. Further research efforts are required to establish the relationship between these biomarkers and presence of schistosome-related morbidity as measured using point-of-care indicators in larger cohorts of populations chronically exposed to schistosome infections.

In summary, the findings of this thesis highlight the need for the refinement of existing diagnostic methods for accurate detection of infection and morbidity in children. This will enable appropriate and timely intervention strategies, aimed at improving the current and future health of preschool aged-children to be implemented. The findings presented here will aid researchers and other stakeholders in making informed choices about intervention tools for control programmes targeting young children.

Lay Summary

This thesis focused on urogenital schistosomiasis, an important, but neglected, infectious tropical disease affecting over 100 million people, mainly in sub-Saharan Africa. Infection and disease are controlled by treatment of infected individuals with the antihelminthic drug, praziquantel. Until recently, schistosome-related morbidity had been thought to be only dependent on infection intensity and most studies have focused on older children (≥6 years) and adults. A growing number of studies from Africa have reported high prevalences of infection and morbidity in pre-school children (aged 5 years and below) but currently these children have been excluded from schistosomiasis control programmes. Recent reports suggest that the development of schistosome-related morbidity is influenced by the nature of the induced immune response and its effects on the associated pathology in target organs. Schistosome-associated immunopathology is mediated by tissue damage and pro-inflammatory immune responses, whose markers can be detected serologically. It is therefore considered that such circulating biomarkers may provide a new valuable approach for early diagnosis of schistosome-related morbidity. In this thesis, I assessed ways of improving the diagnosis of infection and morbidity using existing tools, as well as investigated ways of refining the existing drug regimen to improve the current and future health of pre-school children using different statistical methodologies. This thesis focused on children aged 1–10 years of age, comparing the performance of different diagnostic tools applicable in the field and determine the impact of single *vs.* double treatment with praziquantel on the current and future health status of the children.

I confirmed that pre-schoolchildren carry significant schistosome infections, and that most of these infections are missed by the current standard egg count diagnostic method. By using more sensitive serological methods, I was able to demonstrate that the parasitological techniques might misclassify communities for the treatment regimens recommended by the World Health Organization resulting

in fewer treatments than those required. Furthermore, when evaluating current point-of-care diagnostic tools for schistosome morbidity, I demonstrated that (i) for both pre- and primary school children clinical diagnosis was the least reliable tool, while the urinalysis method was the most reliable, (ii) preschool children carried significant levels of morbidity and (iii) that treatment not only effectively reduced infection levels but also significantly reduced morbidity levels both within 12 weeks and 12 months following initial treatment. My results also revealed the involvement of serological biomarkers in the biological processes of acute inflammation these results suggest that these biomarkers can help in identifying the initial stages of schistosome-related pathology in young children.

In summary, the findings of this thesis highlight the need for the refinement of existing diagnostic methods for accurate detection of infection and morbidity in children. This will enable appropriate and timely treatment strategies, designed to improve the current and future health of preschool children to be implemented. The findings presented here will aid researchers and other stakeholders in making informed choices about intervention tools for control programmes targeting young children. Although I have focused on paediatric schistosomiasis, the issues raised by my thesis are also relevant in older populations. Firstly, accurate diagnosis of infection using more sensitive tools is important in older people suffering from chronic but inactive schistosomiasis for successful mass drug administration programmes. Secondly, poor markers of morbidity mean that pathology associated with schistosomiasis is not detected early enough when it can still be reversed by chemotherapy. Thus, a better understanding of morbidity due to schistosomiasis is still desired in older populations for improved control strategies. Lastly, poor indicators of improved health following antihelminthic treatment make it difficult to fully appreciate the efforts of using praziquantel as a control tool beyond the transient reduction of infection levels in endemic areas.

Declaration

I declare that this thesis has been composed by myself, that the work presented here is my own unless stated otherwise, and that this work has not been submitted for any other degree or professional qualification. The data considered in this thesis were obtained from immuno-epidemiological studies in Zimbabwe as part of a major project: "Health Benefits of Repeated Treatment in Paediatric Schistosomiasis" conducted in collaboration with the University of Zimbabwe (UZ), the National Institute of Health Research, Zimbabwe (NIHRZ), and the University of Edinburgh, UK. I was involved in most of the field work that consisted of collection of urine samples for parasitology and urinalysis, data entry, coding and verification in the field to ensure good-quality data. Blood collection for immunological assays was facilitated by experienced local nursing staff and study clinicians. Antihelminthic treatment with praziquantel offered to compliant children, administered by the local physician. Laboratory based work, including measurement of serological biomarkers of inflammation, antibody and plasma cytokine assays was conducted by other members of the Parasite Immuno-epidemiology Group fully acknowledged in this thesis (http://pig.bio.ed.ac.uk/people/edinburgh/). I have undertaken all the statistical analysis presented in this thesis and all the publications included in this thesis are my own work.

Welcome M. Wami
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an understanding of many of the biological principles and techniques presented here. Our lengthy discussions about statistics were fascinating and am happy to hear that you are now enjoying statistical modelling. A particular thanks to Ian Handel for the fruitful Bayesian Modelling discussions we had.

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Acronyms and abbreviations

ANOVA	Analysis of variance
APC	Antigen-presenting cell
CAA	Circulating anodic antigen
CAP	Cercarial antigen preparation
CCA	Circulating cathodic antigen
CHI3L1	Chitinase 3-like-1 protein
CR	Cure rate
CRP	C-reactive protein
DALYs	Disability-adjusted life years
ELISA	Enzyme-linked immunosorbent assay
EPI	Expanded Program on Immunization
ERR	Egg reduction rate
GLM	General linear model
GLMM	Generalized linear mixed model
HIV	Human immunodeficiency virus
Ig	Immunoglobulin
IL	Interleukin
IFN	Interferon
MCMC	Markov chain Monte Carlo
MDA	Mass drug administration

MRCZ	Medical Research Councilof Zimbabwe
MRPP	Multiple response permutation procedure
MANOVA	Multivariate analysis of variance
NIHRZ	National Institute of Health Research Zimbabwe
NMDS	Non-metric multidimensional scaling
NTD	Neglected tropical disease
PZQ	Praziquantel
RMS	Root-mean-square error
SCI	Schistosomiasis Control Initiative
SD	Standard deviation
SE	Standard error
SEA	Soluble egg antigen
SEM	Structural equation modelling
SLPI	Secretory leukocyte protease inhibitor
STH	Soil-transmitted helminths
sTfR	Serum transferrin receptor
SWAP	Schistosome adult worm
Th	T helper cell
TNF	Tumour necrosis factor
Treg	T (helper) regulatory cell
UACR	Urine Albumin-to-Creatinine-Ratio
UNICEF	United Nations Childrens Fund
UZ	University of Zimbabwe
WHA	World Health Assembly
WHO	World Health Organization

Chapter 1

General introduction

1.1 Background

Schistosomiasis is an important, but neglected infectious tropical disease caused by parasitic helminths of the genus *Schistosoma*. It affects over 230 million people worldwide (Colley *et al.* 2014; Vos *et al.* 2012), mostly poverty stricken communities with limited access to clean water and inadequate sanitary facilities (WHO 2012). In endemic areas, children carry the heaviest burden of infection and due to the chronic nature of the infection and continued susceptibility to re-infection, they can remain infected for most of their lives (Dunne & Mountford 2001; Mutapi *et al.* 2008; Stothard *et al.* 2011; WHO 2002). Consequently, they are subject to schistosome-related complications including growth retardation, anaemia, reduced physical activity, poor cognition and memory, and end-stage organ damage such as bladder and kidney disorders (Fenwick *et al.* 2009; King 2007; King & Dangerfield-Cha 2008; Odogwu *et al.* 2006). When adequately treated during childhood with the antihelminthic drug of choice praziquantel (PZQ), symptoms of disease can be reversed and development of severe morbidity alleviated (Bundy *et al.* 2013; King 2007; WHO 2002).

In several countries currently implementing schistosome control programmes, the control strategies follow the directive by the World Health Assembly resolution (WHA54.19) in 2001 (WHO 2001*a*, 2002). This involves regular school-based deworming using PZQ aimed at reducing morbidity, promoting school-child health and improving the cognitive potential of children (WHO 2002, 2010). A growing number of studies from Africa have shown that preschool-aged ($\leq$ 5 years) children are at a high risk of schistosomiasis (Dabo *et al.* 2011; Mutapi *et al.* 2011; Odogwu

et al. 2006; Stothard *et al.* 2011). These findings and concerted efforts led to a major recommendation by the World Health Organization (WHO) in 2010, stating that preschool-aged children should be considered for treatment through regular health services as well as in schistosome control programmes (WHO 2011*a*). These recent developments in schistosome control policy have heightened the need for a clear knowledge on optimal strategies required to improve the effectiveness of interventions targeting this age group.

Among other reasons, preschool-aged children have been excluded from schistosome control programmes because infection had been underestimated in this age group and the resulting morbidity is still poorly defined or quantified. The current thesis seeks to increase the understanding of the schistosome infection and morbidity burden in children aged 1–5 years, and thereby contribute to the planning and implementation of improved control programmes targeting this age group using currently available intervention tools. The performance of available rapid diagnostic tools in addition to using egg counts (widely acceptable as the standard diagnostic technique) are investigated in children aged 1–5 years and compared to primary school-aged (6–10 years) children and their implications for control elucidated. To date, the performance of the point-of-care morbidity diagnostic tools has not yet been evaluated in preschool-aged children. As a consequence, this can have negative impacts on the assessment of the effectiveness of control programmes and thus their prioritization and sustenance within ministries of health in affected countries, often with small health budgets, and other stakeholders.

To address this knowledge gap, I seek to determine the utility of several available diagnostic tools in identifying markers of morbidity associated with schistosome infections in preschool children compared to children aged 6–10 years old, who are the current specific targets of most schistosome control programmes. These would provide an invaluable tool for diagnosis of schistosome-related morbidity, and therefore aid timely treatment. The benefits of PZQ treatment in older children (aged ≥6 years) have already been documented (Agnew-Blais *et al.* 2010; Magnussen *et al.* 1997; Midzi *et al.* 2008). Additionally, in this thesis I investigate the effects of PZQ treatment on health and morbidity measures in preschool-aged children and determine whether the effects of chemotherapy are age-dependent by comparing the findings to children aged 6–10 years old.

The findings of this thesis provide an initial operational recommendation for

future studies on the subject, as well as giving further insights into the health benefits of antihelminthic treatment in preschool-aged children. As such, these findings are important for practitioners, policy makers and stakeholders involved in the control of schistosomiasis and timely because of the current global drive to address the health inequity created by the paucity of information on the disease in infants and preschool children.

1.2 The schistosome life cycle

The main disease causing schistosomes infecting humans are *S. haematobium*, *S. mansoni* and *S. japonicum*. Other species that can infect humans but are rarely reported causes of infection include *S. mekongi* and *S. intercalatum* (Gryseels *et al.* 2006). The intermediate hosts for *S. haematobium* and *S. mansoni* are aquatic freshwater snails that prefer standing or slow-moving water bodies. *S. haematobium* is transmitted by snails of the genus *Bulinus* and causes urogenital schistosomiasis. *S. mansoni* is transmitted by snails of the genus *Biomphalaria* and causes intestinal and hepatic schistosomiasis. The intermediate hosts of *S. japonicum* are amphibian type of snails of the genus *Oncomelania*, also found on moist ground or plants, causing intestinal and hepatosplenic schistosomiasis (Colley *et al.* 2014; Gryseels *et al.* 2006).

Humans acquire schistosomiasis through contact with snail-infested waters whilst performing domestic or occupational duties and through recreational activities (King & Dangerfield-Cha 2008). The life cycle of human schistosomes occurs in stages, and the species differ in their intermediate host (snails), the final location in the human host (definitive host), and the size and shape of the eggs they produce as illustrated in Figure 1.1. When eggs excreted (through urine or stool) from infected individuals come in contact with water they hatch, releasing miracidia which seek and infect the intermediate snail host. The miracidia multiply asexually into sporocysts and later develop into free-living cercariae. The cercariae are released from the snail and seek a definitive host, where they develop into adult worms in the hepatic portal vein of the liver after 4–6 weeks (Gryseels *et al.* 2006).

Fully grown schistosomes can range from 7–20 mm in length and have two terminal suckers, a blind digestive tract and reproductive organs. The mature adult male and female worms mate and migrate to their final destination, either

in the venous plexus of the bladder (for *S. haematobium*) or mesenteric veins of the intestine (for *S. mansoni* and *S. japonicum*) where the females lay hundreds of eggs per day. The schistosomes can live in their human host for an average of 3–10 years, although some species can live for more than 30 years, feeding on blood and globulins through anaerobic glycolysis. The eggs penetrate into the lumen of the intestines or bladder and are then expelled in the faeces or urine respectively, and can remain viable for up to seven days (Colley *et al.* 2014; Gryseels *et al.* 2006).

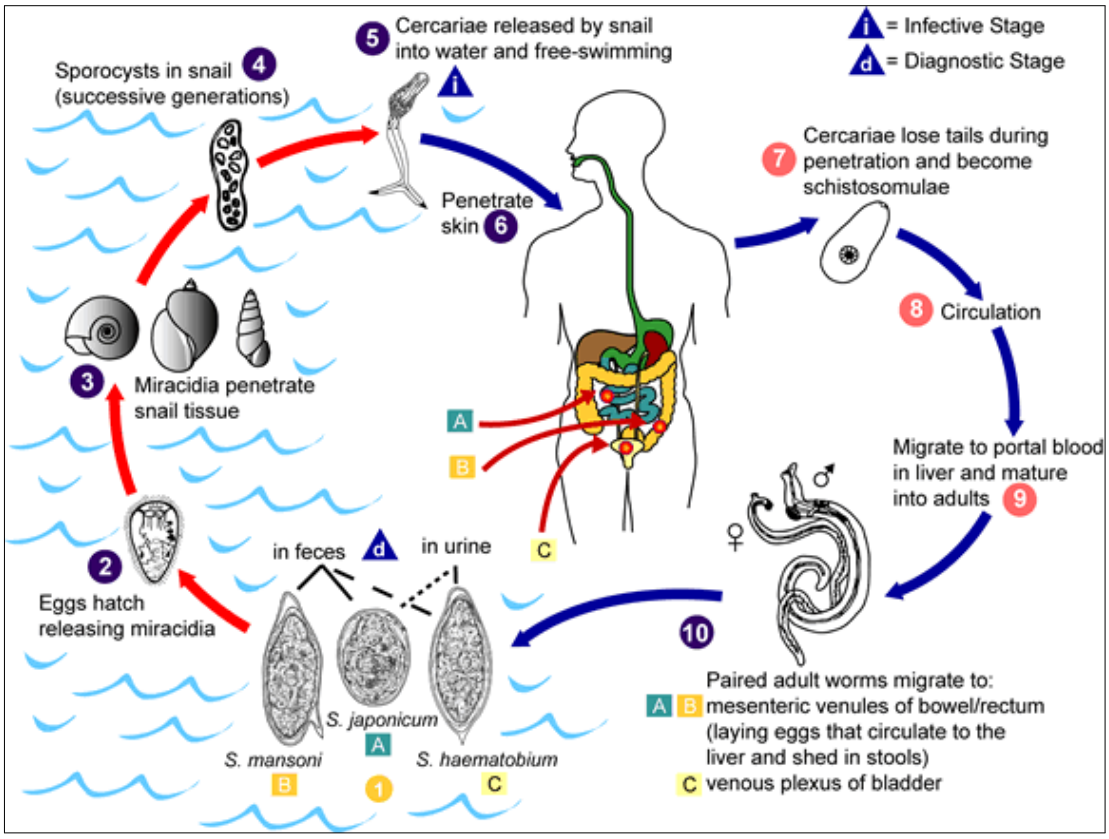


Figure 1.1: The life cycle of schistosomes. Snail infecting, free-living water-borne, and human-dwelling stages of the lifecycle. Adapted from the Centre for Disease Control and Prevention (CDC) online resources: http://www.dpd.cdc.gov/dpdx [Accesed 27/12/2014].

1.3 Epidemiology of schistosomiasis

The geographical distribution of schistosomiasis depends on the ecology of the intermediate snail hosts and their prevalence among freshwater habitats like ponds, natural streams, lakes and man-made water reservoirs and irrigation systems (WHO 2012). Of the estimated 230 million people infected with schistosomiasis globally, 85% of the disease burden is concentrated in sub-Saharan Africa (Colley *et al.* 2014; Engels *et al.* 2002; Lengeler *et al.* 2002; Vos *et al.* 2012). The worldwide mortality due to schistosomiasis is estimated at more than 200, 000 deaths per year (King *et al.* 2005; van der Werf *et al.* 2003) and more than 1.5 million disability adjusted life years (DALYs) are estimated to be lost due to schistosome infections (King 2010; WHO 2008). *S. haematobium* and *S. mansoni*

are the major species commonly distributed throughout Africa (Figure 1.2). *S. haematobium* is also found in the Middle East along the Tigris and Euphrates, and southern parts of the Arabian Peninsula. *S. mansoni* also occurs in the western parts of South America (mainly Brazil), and some parts of the South Caribbean islands. *S. japonicum* occurs in parts of China, the Philippines, and Indonesia (Colley *et al.* 2014; Gryseels *et al.* 2006).

In some countries the prevalence of schistosome infection has been successfully reduced to very low levels, although ultimate elimination of the disease is yet to be confirmed in these areas (WHO 2013). In these endemic regions, children tend to carry the heaviest burden of infection, beginning to accumulate worm burdens as soon as they are old enough to come into contact with water contaminated with cercarial larvae (Mutapi *et al.* 2008; Stothard *et al.* 2011). Children are most vulnerable to infection or re-infection due to their high patterns of exposure such as playing, swimming or fishing in infested waters. Age-related changes in infection levels have been reported in various epidemiological studies (Mitchell *et al.* 2011; Mutapi *et al.* 2007; Woolhouse *et al.* 2000), and these characteristic age-intensity profiles have been associated with the development of acquired protective immunity in older individuals (see section 1.9.3). Reported sex-related differences in schistosome infection prevalences in children are not high, with both male and female children having relatively high water contact patterns (Chipeta *et al.* 2013; Sacko *et al.* 2011; Sow *et al.* 2011).

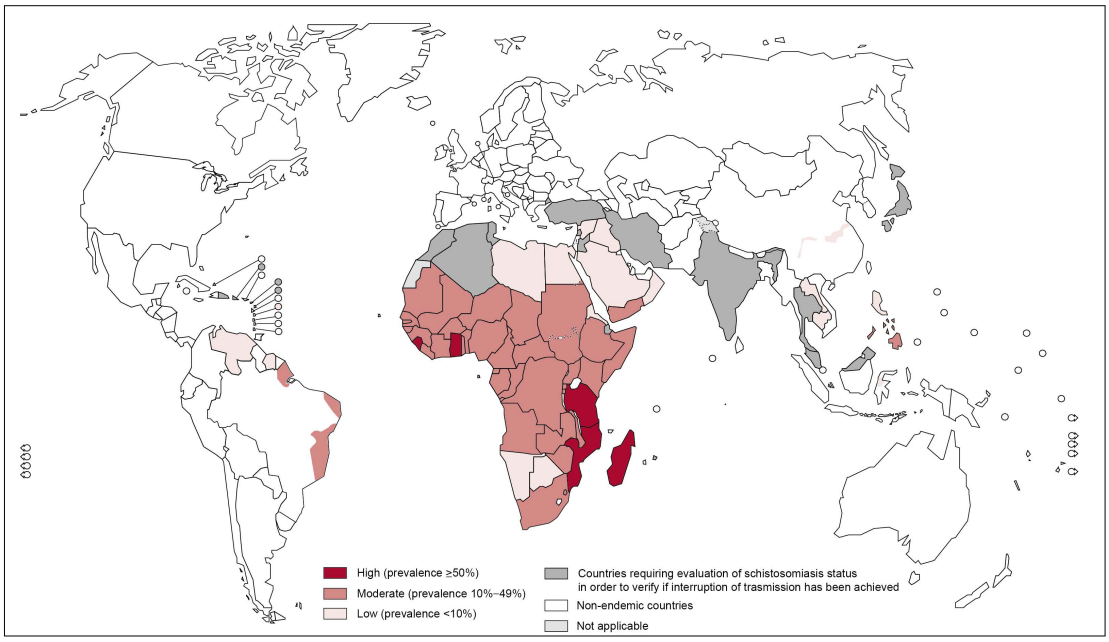


Figure 1.2: Map of the worldwide distribution of schistosomiasis as of 2011. Source from the World Health Organization online resource: http://gamapserver.who.int/mapLibrary/ [Accesed 28/12/2014].

1.4 Pathogenesis of schistosomiasis

The development of schistosome-related morbidity results from the damage caused by the parasite eggs and as a consequence of the host immune reaction to eggs trapped in tissues (Gryseels *et al.* 2006; Smith & Christie 1986). The tissue damage caused by lodged or passing parasite eggs has been associated with an increase in susceptibility to other infections such as bacterial or viral infections (Fairfax *et al.* 2012; Kjetland *et al.* 2008; Mbabazi *et al.* 2011; Pearce & Simpson 1994). The type of host immune response is crucial in determining the pathological changes associated with schistosome infections and the severity of disease (Gryseels *et al.* 2006; Pearce & MacDonald 2002). Antigens from the eggs stimulate granulomatous reactions, mediated by T-cells, eosinophils and macrophages. The chronic inflammatory response leads to collagen formation and fibrosis, causing obstruction to portal blood flow and urine flow, eventually resulting in organ damage (Colley *et al.* 2014; Pearce & MacDonald 2002; Warren 1978). Individual host variations in disease sequelae due to schistosome infections are well documented in the literature, with some people showing more severe

pathological changes compared to others despite a similar burden of infection intensity (Chen & Mott 1989; Isnard *et al.* 2010; Kabatereine *et al.* 2007; Mohamed-Ali *et al.* 1999; van der Werf & de Vlas 2004). More importantly, the development of subtle morbidity due to schistosomiasis has significant impact on the general childhood health, hence the need for timely prevention and other control measures (Ekpo *et al.* 2012*a*; WHO 2002).

1.5 Morbidity due to schistosomiasis

The morbidity due to schistosome infections can be divided into three main stages: migratory, acute and chronic schistosomiasis. The migratory stage is associated with initial skin penetration by cercariae, resulting in a hypersensitivity reaction (swimmer's itch or cercarial dermatitis) that often occurs within a few hours after exposure (Gryseels *et al.* 2006; Lambertucci 2010). Symptoms associated with the acute stage schistosomiasis, also referred to as Katayama syndrome (reviewed recently by Ross *et al.* (2007)) can develop between 4–10 weeks after exposure and is characterized by fever, rash, abdominal pain, lymphadenopathy and eosinophilia (Colley *et al.* 2014). Bloody diarrhea and tender hepatomegaly have also been associated with heavy *S. mansoni* or *S. japonicum* infections at this stage (Lambertucci 2010). These early (migratory and acute) inflammatory reactions are rarely observed in residents of endemic regions (King & Dangerfield-Cha 2008), possibly due to *in utero* sensitization (Rujeni *et al.* 2012). The chronic phase of schistosomiasis is accompanied by a wide variety of symptoms, more general and/or organ-specific, depending on the species (King & Dangerfield-Cha 2008; Smith & Christie 1986) as summarized in Table 1.1.

The reported major chronic forms of schistosome-related morbidity are hepatosplenic (*S. mansoni* and *S. japonicum*) and obstructive urinary tract (*S. haematobium*) disease. Chronic infection with schistosomes in children is associated with iron deficiency anaemia and undernutrition which have significant negative impacts on child growth (WHO 2001*b*). Nutritional deficiencies are caused by the growth and development of the parasite inside the host and the passing of their eggs through the host causing tissue damage and inflammation. The damage to the organ tissues causes blood and iron loss resulting in iron deficiency anaemia (Friedman *et al.* 2005). Malnutrition and anaemia are associated with impaired child growth, reduced physical fitness, and poor cognitive

and mental development (Haas & Brownlie 2001; Shaw & Friedman 2011). In addition, the resulting tissue damage inflicted by the parasite eggs can result in increased susceptibility to co-infections such as bacterial and viral infections (Knopp *et al.* 2013).

The genital manifestations of *S. haematobium* infection can have an impact on the reproductive health in adulthood (Feldmeier *et al.* 1999; Nour 2010). In women, schistosome infection can lead to complications in the vulva or fallopian tube, making them more vulnerable to HIV infection (Kjetland *et al.* 2008, 2006; Mbabazi *et al.* 2011), and these effects have been recently reviewed in detail elsewhere (Kjetland *et al.* 2012). In males, *S. haematobium* infection has been associated with testicular and ejaculatory pain (Leutscher *et al.* 2000*a*; Lewis *et al.* 1996). In addition, delayed or lack of treatment can result in more severe and irreversible forms of disease including bladder cancer, kidney failure, and liver or spleen damage which may eventually result in death (King & Dangerfield-Cha 2008; Olveda *et al.* 2014; Smith & Christie 1986).

Table 1.1: Type of morbidity or pathology associated with schistosome infections in humans. Species-specific symptoms and general conditions associated with schistosomiasis.

S. haematobium	***S. mansoni***	***S. japonicum***	**General morbidity**
Haematuria	Periportal fibrosis	Periportal fibrosis	Anaemia
Dysuria	Portal hypertension	Portal hypertension	Malnutrition
Genital lesions	Hepatosplenomegaly	Splenomegaly	Stunted growth
Renal failure	Abdominal pain	Hepatic disease	Fatigue
Bladder cancer	Bloody diarrhoea	Abdominal pain	Poor memory development
Supra-pubic pain		Bloody diarrhoea	Impaired cognition
Bladder calcification			Reduced physical fitness
Urinary tract infections			Susceptibility to co-infections

Sources: Colley *et al.* (2014); King & Dangerfield-Cha (2008); Kjetland *et al.* (2012); Smith & Christie (1986); Warren (1978); Webster *et al.* (2009).

1.6 Treatment and control of schistosomiasis

Several drugs have been developed and used on a large scale for the treatment of schistosomiasis. Treatment is judged to be successful when it cures the infection either by completely eradicating the schistosomes from the host, or when there is a significant reduction in egg counts (in stool or urine), or a significant decrease in prevalence of infection and related morbidity (Forrester & Pearce 2006). Older drugs that have been successfully used in the treatment of schistosomiasis include metrifonate (Davis & Bailey 1969) and oxamniquine(Sleigh *et al.* 1981). Praziquantel (PZQ), introduced into clinical practice in the late 1970s, is currently the drug of choice for schistosomiasis treatment both at clinical and community based level because of its high efficacy, low toxicity and ease of administration (Cioli Livia *et al.* 1995; Danso-Appiah *et al.* 2008; Reich *et al.* 1998; WHO 2002).

Metrifonate (O,O-dimethylhydroxy-2,2,2-trichlorethyl-phosphonate) is only effective against *S. haematobium* (Davis & Bailey 1969; Feldmeier *et al.* 1982), and is administered in three doses of 10 mg/kg two weeks apart (Reich *et al.* 1998). The known pharmacological action of metrifonate is its inhibitory effect on worm cholinesterases (a group of enzymes associated with the parasite muscle tissue and signal transduction), thereby inducing paralysis of the schistosome (Abdi *et al.* 1995; Arnon *et al.* 1999; Bloom 1981). Previous studies suggest that *S. haematobium* may be more susceptible to metrifonate than *S. mansoni* because of much higher levels of cholinesterase activity in its tegument (Abdi *et al.* 1995; Camacho *et al.* 1994). Metrifonate generally has a lower reported efficacy than praziquantel due to poor compliance associated with the treatment schedule, and thereby making it less ideal for mass treatment programmes for *S. haematobium* in endemic areas (Abdi & Gustafsson 1989; Danso-Appiah *et al.* 2008).

Oxamniquine is a semi-synthetic tetrahydroquinoline derivative, and it acts by inhibiting the nucleic acid metabolism of the parasites, resulting in contraction and paralysis of the worms and eventually promoting damage of the dorsal tegument, and death of the schistosomes (Abdi *et al.* 1995). Oxamniquine is only effective against *S. mansoni*, and the effective dose varies between 15 mg/kg and 60 mg/kg administered over two to three days (Ferrari *et al.* 2003; Kilpatrick *et al.* 1982; Sleigh *et al.* 1981). The drug is more effective against male than female schistosomes and has no effect in other human schistosome species. Over

the past two decades, oxamniquine has been successfully used in the national control programmes of *S. mansoni* in South America, mainly in Brazil (Hotez *et al.* 2006). Although highly efficacious, oxamniquine has more reported side-effects and is more expensive compared to praziquantel (Ferrari *et al.* 2003).

Praziquantel is a pyrazinoisoquinoline derivative, well known for its biological activity against schistosomes (Kim *et al.* 1998). The drug is administered at a recommended single oral dosage of 40mg/kg and costs approximately US $0.08 per tablet (WHO 2002). PZQ has strong effects on the physiology and morphology of the schistosomes, by modifying intracellular voltage-gated calcium ion (Ca^{2+}) levels in the adult worm. Immediate exposure of schistosomes to the PZQ drug disrupts the homeostasis of Ca^{2+} thereby triggering a cascade of events that induce a rapid and sustained contraction of the worm's musculature (paralysis) and damage to the tegument, which subsequently exposes the parasite antigens on the surface of the worm to attack by the host immune system (Doenhoff *et al.* 2008; Greenberg 2006). The mechanism of action of PZQ, its pharmacological properties, health benefits and side effects have been reviewed in detail elsewhere (Bundy *et al.* 2013; Cioli Livia *et al.* 1995; Dayan 2003; Doenhoff *et al.* 2008; Greenberg 2006). Evidence from different studies has shown that treatment also alters the host immune response (see section 1.9.6), resulting in changes in the levels of schistosome-specific antibody responses (Jiz *et al.* 2009; Mutapi *et al.* 2005, 2003). In addition, it has been shown that the exposure of previously hidden antigens to the host immune system following the death of the parasites due to the effects of praziquantel can boost antibody responses associated with protection against re-infection, subsequently leading to the development of acquired immunity to the parasite (Mitchell *et al.* 2012; Mutapi *et al.* 2005). PZQ is highly efficacious, with cure rates of more than 80% reported within 6–8 weeks following treatment (Tchuente *et al.* 2004). Furthermore, PZQ is effective against all the major schistosome species and thus advantageous for the control of mixed infections (Koukounari *et al.* 2010; Meurs *et al.* 2012). PZQ has been recently shown to be safe for infants and preschool children (aged ≤5 years old) (Mutapi *et al.* 2011; Sousa-Figueiredo *et al.* 2012; Stothard *et al.* 2011). These recent findings have contributed towards a major policy change by the WHO in 2010, recommending PZQ treatment for children aged 5 years and below through regular health services and on-going schistosome control programmes (WHO 2011*a*).

Currently, most schistosome control programmes focus on preventive chemother-

apy with PZQ to reduce infection and prevent the development of severe morbidity, and thereby improving the health of the infected individuals and communities (WHO 2002, 2010). Since PZQ chemotherapy targets mature adult worms and the risk of re-infection is high in endemic areas, repeated treatment of the target populations at regular intervals is necessary for effective control (WHO 2002). Recommended additional measures for an integrated effort aimed at achieving sustainable control of schistosomiasis in endemic regions include improvements in clean water supply and sanitation, health education, and snail control (WHO 2013).

To support the current control measures, scaled-up efforts have been geared towards development of a vaccine for schistosomiasis (Capron *et al.* 2002). However, there is no available licensed schistosome vaccine to date and the most promising vaccine candidate Bilhvax, based on the 28-kDa glutathione S-transferase from *S. haematobium* (Sh28GST) is in phase III clinical trials (Riveau *et al.* 2012). Since praziquantel is only effective against adult worms, and although not yet clinically proven, fears of schistosome resistance to the drug have also been raised (noted by Doenhoff *et al.* (2008)). Thus, in an era of intensified control, there is need to explore other alternative therapies. Artemisinin and its derivatives have been found efficacious against immature worms and hence may prove to be a potential treatment alternative targeting the larval stages of the parasite not yet susceptible to praziquantel (Doenhoff *et al.* 2008; Liu *et al.* 2011, 2014). The antihelminthic activity of mirazid, a derivative of myrrh has been suggested in some previous studies (Barakat *et al.* 2005; Ebeid *et al.* 2005; Ramzy *et al.* 2010). Further research studies on mirazid as a potential antischistosomal drug against the developmental stages of the worms are still needed.

1.7 Diagnosis of schistosome infection

Accurate detection of infection and quantification of the morbidity burden is important for the management of schistosomiasis both at a clinical and community level. Correct diagnosis of infection will facilitate timely treatment of infected individuals and prevent the development of severe morbidity associated with chronic schistosomiasis (King 2007; Polman 2000; WHO 2007). In recent years, there has been advances in the development of new methods aimed at improving the detection of schistosome infections. The currently available techniques for

the diagnosis and quantification of infection levels can be divided into two main categories: direct and indirect diagnostic methods. The direct method is the current standard diagnostic technique recommended by the WHO, and involves the parasitological detection of parasite eggs in stool and/or urine (Katz *et al.* 1972; Mott *et al.* 1982). The parasitological diagnostic technique has high specificity and does not require costly equipment or advanced technical skills, hence relatively easy to implement on a large scale under field conditions. However, day-to-day variations in parasite egg excretion makes it difficult to determine infection intensity levels with high precision using parasitology (Doenhoff *et al.* 2004; Engels *et al.* 1996). To improve the accuracy of the parasitological technique, repeated examinations of urine/stool samples is recommended, however collection of several specimens is not always feasible in large-scale studies for logistic reasons and the associated costs (Mutapi 2011; Stothard 2009). In addition, the parasitological technique is less sensitive in detecting light infections, and does not diagnose pre-patent and/or single-sex infections where there is no egg-production, but still important for transmission (Bergquist *et al.* 2009; Doenhoff *et al.* 2004; Mutapi 2011; Smith & Christie 1986).

With recent advances in technology, several indirect methods aimed at improving the diagnosis of schistosome infection under clinical or community settings have been developed and validated in older children and adult populations. These indirect methods rely on biological or biochemical disease markers and other clinical manifestations associated with schistosomiasis (Ambrosio & De Waal 1990; Polman 2000; Smith & Christie 1986). Dipstick detection of microhaematuria (Adesola *et al.* 2012; King & Bertsch 2013) and self-reported questionnaires about symptoms associated with schistosomiasis, for example, haematuria and dysuria (Clements *et al.* 2008; Lengeler *et al.* 2002) are examples of available indirect tools that have been successfully used for large-scale screening of urogenital schistosomiasis in the field. Serological detection of parasite-specific antibodies has also been used as an indirect indicator of the presence of infection in previously untreated individuals and communities (Alarcón de Noya *et al.* 2007; Smith *et al.* 2012; Sorgho *et al.* 2005; Wami *et al.* 2014; Xu *et al.* 2014). The performance and applicability of antibody detection as a diagnostic tool has been evaluated in several immuno-epidemiological studies (Dawson *et al.* 2013; Kanamura *et al.* 2002; Kinkel *et al.* 2012; Smith *et al.* 2012) and has been reviewed in detail by Doenhoff *et al.* (2004). In particular, the serological

detection of IgM antibody responses against soluble egg antigens (SEA) has been identified as an attractive diagnostic tool due to its high sensitivity relative to the parasitological technique, and thus is highly suitable for improved detection of light infections (Doenhoff *et al.* 1992; Turner *et al.* 2004). Furthermore, a positive association of SEA IgM with infection levels (intensity and prevalence) has been demonstrated (Dawson *et al.* 2013; Stothard *et al.* 2011; Wami *et al.* 2014; Woolhouse *et al.* 2000).

Other indirect methods characterized to detect light infections include the immunological detection of circulating adult-worm derived antigens in urine: circulating anodic antigen (CAA), and circulating cathodic antigens (CCA) (Dawson *et al.* 2013; Midzi *et al.* 2009; Stothard *et al.* 2006; van Lieshout *et al.* 2000). The applicability of these antigen assays for large-scale screening has been hindered by its cost and lack of sensitivity in detecting *S. haematobium* infections (Shane *et al.* 2011; Stothard *et al.* 2009). DNA detection has been identified in recent studies as a promising future tool for the diagnosis of schistosome infections (reviewed by Cavalcanti *et al.* (2013)), however its applicability is still yet to be fully investigated.

1.8 Immunity

1.8.1 Immune responses

In order to survive, the host must mount an effective immune response against a wide variety of pathogens such as parasites, viruses, and bacteria (Murphy 2012). In addition, it is also important for the immune responses to be regulated, failure to which may lead to autoimmunity, a condition where the immune system starts attacking the healthy cells (Dunne & Cooke 2005; Klion & Nutman 2002; Murphy 2012). The host immune system can be divided into two components that complement each other to coordinate a given response against invading pathogens: the innate, and adaptive (or acquired) immunity (Murphy 2012; Sompayrac 1999; Warrington *et al.* 2011). In brief, the innate immune response is the first line of defense to combat infection but does not result in a lasting immunological memory and is non-specific to any individual pathogens. On the other hand, acquired immunity takes time to develop, is highly specific against a particular invading pathogen or its antigens and does result in immunological memory.

Acquired immunity can be further separated into two forms: the humoral (or antibody-mediated) and cellular (or cell-mediated) immunity (Murphy 2012). Humoral immunity deals with infectious agents in the blood and body tissues, i.e., it is directed against extracellular pathogens (Mak & Saunders 2006). It involves secretion of antibodies and is mediated by B-cells. Antibodies, also known as immunoglobulins (Ig) circulating in serum are classified into five major categories: IgA, IgD, IgG, IgE, and IgM, with distinct biological activities in protection against infections (Woof & Burton 2004). Antibodies target the pathogens by binding onto their surface and thereby flagging them for destruction through pathogen and toxin neutralization, and through promotion of phagocytosis by other cells such as macrophages (Warrington *et al.* 2011). Cellular immunity deals with body cells that have been infected, hence it is for defense against abnormal cells and intracellular pathogens (Mak & Saunders 2006). The cellular immune response involves the activation of phagocytes, antigen-specific cytotoxic T-cells and the release of cytokines in response to an antigen, and is mediated by T-cells (Murphy 2012; Warrington *et al.* 2011). Cytokines secreted by many cells types interact with each other and with other cells of the immune system in order to regulate the host response to infection. Some cytokines inhibit the growth of other cells, hence controlling the expansion of infected cells. Other cytokines have a direct effect on B-cells, controlling their maturation, proliferation, antibody secretion and isotype switching (Murphy 2012). Plasma levels of systemic cytokines which have been previously reviewed and shown to mediate inflammatory and anti-inflammatory responses associated with schistosome infections include tumour necrosis factor (TNF)-α, transforming growth factor (TGF)-β, interferon (IFN)-γ, and interleukins (IL)-4, IL-5, IL10, IL-13 (Caldas *et al.* 2008; Coutinho *et al.* 2006; Imai *et al.* 2011; Macdonald *et al.* 2002; Milner *et al.* 2010; Mutapi *et al.* 2007).

1.8.2 Immunological memory and vaccination

Immunological memory is an important consequence of the acquired immune response, resulting in protective immunity. Having been exposed to an infectious agent, the host will be able to invoke a quicker and stronger immune response on subsequent exposure to the same infectious agent (Crotty & Ahmed 2004). The secondary immune response is characterised by a more vigorous and earlier abundant production of antibodies than in the primary response. This rapid

immune response can either prevent infection completely or reduce the severity of the associated morbidity (Crotty & Ahmed 2004; Zanetti & Croft 2001). Several conditions to ensure maintenance of immunological memory have been suggested in literature and still remain an interesting area of research (Bourke *et al.* 2011; Crotty & Ahmed 2004; Zanetti & Croft 2001; Zielinski *et al.* 2011). Immunological memory can be sustained through periodic re-exposure to the antigen, as this helps to maintain a high level of antibodies and serves as a natural booster for the immune system. In the absence of re-exposure to the antigen, long-lived plasma cells can also maintain immunological memory through continuous secretion of antibodies (Crotty & Ahmed 2004). Immunological memory can be induced by vaccination. The components of a vaccine contain antigens which may be an inactivated, attenuated form of the pathogen, or purified components of the pathogen that stimulates an immune response. This will prepare the host immune system to induce a stronger and more rapid response on encounter with the antigen later in life (Zielinski *et al.* 2011).

1.9 Immunobiology of schistosomiasis

1.9.1 Schistosome antigens

As highlighted in the preceding sections, most of the pathology associated with schistosome infections is a consequence of the host immune response to antigens of the parasite eggs (Burke *et al.* 2009; Wynn *et al.* 2004). The schistosome antigens circulating inside the host can be classified according to the life cycle stage of the parasite: cercarial, schistosomula, adult worm (tegument or gut-associated), and egg antigens (Curwen *et al.* 2004; Lu *et al.* 2012). The life cycle stage-specific immune responses are commonly assessed using crude homogenate preparations, namely: cercarial antigen preparation (CAP), soluble adult worm preparation (SWAP) and schistosome soluble egg antigen (SEA). These preparations consist of the crude parasite molecules representing the wide range of parasite antigen molecules present during natural infection (Bourke *et al.* 2011; Curwen *et al.* 2004). Detection of circulating parasite-specific antibodies in serum can be a useful epidemiological tool for the diagnosis of pre-patent, single-sex, or light infections, and quantifying levels of exposure to the parasite (Smith *et al.* 2012; Wami *et al.* 2014; Woolhouse *et al.* 2000).

The surface of the cercariae is covered by a layer of carbohydrate-rich glycocalyx (Nanduri *et al.* 1991). Carbohydrate antigens released by the penetrating cercariae, as they lose their outer layer to transform to schistosomula and migrate to the lungs invokes a strong host immune response to facilitate the clearance of the parasite at an early stage (Harrop *et al.* 2000; Xu *et al.* 1994). The tegument is the outer layer covering the schistosomes which forms an interface between the parasite and the host (Abath & Werkhauser 1996). Adult worm schistosome antigens can be associated with the tegument or gut of the parasite, both are involved in the feeding mechanisms of the parasite from the bloodstream of the host (Skelly *et al.* 2014). The tegumental antigens consist of cytoplasmic and membrane bound molecules of the parasite. Experimental evidence suggests that not all live adult worm tegument-associated antigens are exposed to the immune system during natural infection (Curwen *et al.* 2004; Harrop *et al.* 2000; Silva *et al.* 2004). This reduced surface antigenicity enables the adult worms to escape immune recognition (Gryseels *et al.* 2006; Pearce & MacDonald 2002), and hence survive longer inside the host (see section 1.9.4). Since schistosomes have a blind digestive system (i.e. have no anus), the waste products are regurgitated into the blood stream of the host (Skelly *et al.* 2014). Thus, the gut-associated antigens are the major circulating group of adult worm antigens and can be detected in serum and urine of infected individuals (van Lieshout *et al.* 2000). The most studied gut-associated circulating antigens include CAA and CCA (introduced in section 1.7), and these have been investigated recently as additional tools for serological diagnosis of the major human schistosome infections in endemically exposed populations (Dawson *et al.* 2013; Lu *et al.* 2012; Midzi *et al.* 2009). Schistosome parasite eggs are highly immunogenic and act as the main source of inflammatory responses leading to the development of granulomatous lesions (Pearce 2005*a*). During chronic infection which coincides with egg production, cytoplasmic proteins are secreted from various cells of the parasite eggs, against which a strong immune response is initiated. The nature and intensity of the host immune response against egg antigens (detailed in section 1.9.2) play an important role in the severity of disease (Pearce 2005*b*).

1.9.2 Immune responses to schistosomes

The circulating antigens during the acute and chronic phases of schistosome infections induce strong host immune responses that often cause a wide variety of

pathological outcomes. Thus, it is important to understand the factors involved in the immune response to schistosomes, as these can precede schistosome-immune mediated pathology, and hence can be informative indicators of risk of current and future disease. The mechanisms of the immune response to schistosomiasis is a complex balance between T helper 1 (Th1) and Th2 cells, and it also involves antibody-mediated immune responses (Caldas *et al.* 2008; King *et al.* 2001; Pearce & MacDonald 2002; Zhang & Mutapi 2006). In addition, the regulatory immune response (led by T regulatory cells) has been shown to play an important role in modulating the detrimental effects of immune responses during infection (Milner *et al.* 2010; Nausch *et al.* 2011; Watanabe *et al.* 2007).

During the early stages of infection, the host immune response stimulated by the penetrating cercariae and the migrating schistosomula antigens is dominated by the Th1 type of immune response. The antigen presentation from these stages stimulates secretion of pro-inflammatory cytokines such as TNF-α, IFN-γ, IL-1, IL-2, and IL-6 by activated CD4+ T-lymphocytes, associated with early granuloma formation (Olveda *et al.* 2014; Pearce & MacDonald 2002). The hypersensitivity reaction to cercarial antigens manifests as a skin rash (swimmers itch or cercarial dermatitis), that sometimes persists for days as pruritic lesions (Gryseels *et al.* 2006). Katayama fever is a clinical manifestation of acute schistosomiasis that occurs as an immune reaction to the migrating schistosomulae (Colley *et al.* 2014; Gryseels *et al.* 2006). The symptoms associated with the acute stage of infection are commonly reported among migrants and tourists who get exposed to the parasite for the first time whilst visiting endemic regions, since they have not yet developed immunologic tolerance (Lambertucci 2010). As infection persists and egg laying by mated adult female worms commences, the dominant immune response gradually shifts from a Th1 to a Th2 type of response against the parasite egg antigens. The Th2 cells elevate the secretion of cytokines such as IL-4, IL-5, IL-10 and IL-13, accompanied by high production of eosinophils and contributing to granuloma formation and maturation (Magalhães *et al.* 2004; Olveda *et al.* 2014). In recent studies, IL-10 has been characterized as an immuno-regulatory cytokine during schistosome infections which modulates Th1 and Th2 effector cytokine expression (Caldas *et al.* 2008; Corrêa-Oliveira *et al.* 1998), and thus provides a balance in the immune responses (Burke *et al.* 2009; Maizels & Yazdanbakhsh 2003).

The antibody-mediated response plays a crucial role in effector or regulatory

immune response mechanisms against parasite-specific antigens and occurs during both the acute and chronic stages of schistosomiasis (Caldas *et al.* 2008; Eberl *et al.* 2001). IgM antibodies appear early in the course of an infection, and often re-appear on further exposure and have been shown to increase with schistosome infection levels (Imai *et al.* 2011; Kwame Nyame *et al.* 1997; Mutapi *et al.* 1997; Ndhlovu *et al.* 1996). In addition, they are often found to bind to specific antigens (Warrington *et al.* 2011). These properties make IgM a useful additional sensitive diagnostic tool for schistosome infections in untreated populations, as demonstrated in *S. haematobium* field studies (Stothard *et al.* 2011; Wami *et al.* 2014; Woolhouse *et al.* 2000). IgE has been associated with host immune protection against schistosome infection (Jiz *et al.* 2009; Zhang & Mutapi 2006). Circulating levels of IgA and IgG1 have also been implicated in the development of protective immunity against re-infection in older individuals, whilst IgG4 has been associated with susceptibility to infection. In addition, ratios of IgE:IgG4 and IgG1:IgA have been shown to increase with age (described in section 1.9.5), also associated with the development of resistance to infection (Butterworth 1998; Grogan *et al.* 1997; Mutapi *et al.* 2011, 1997). The switch between different antibody isotypes has been reported in schistosomiasis. The progression of infection from the acute to the chronic phase is characterized by a switch from IgM to IgE antibody-mediated responses. In addition, changes in antibody production after antihelminthic treatment associated with resistance to infection have been reported, characterized by a shift from IgA to IgG1 antibody production (Mutapi *et al.* 1998, 1999; Oliveira *et al.* 2005).

1.9.3 Protective immunity

Epidemiological studies have shown that in high transmission areas the infection intensity follows a characteristic convex curve with age, rising rapidly in early childhood as children accumulate worms, peaking between the ages of 9–15 years old, and subsequently declining in adulthood (Chandiwana & Woolhouse 1991; Mitchell *et al.* 2011; Woolhouse 1994). In addition, lower levels of re-infection following chemotherapy among adults compared to children have been reported (Pinot de Moira *et al.* 2010; Roberts *et al.* 1993). These age-related changes in infection levels (see Figure 1.3) have been associated with the development of acquired protective immunity in older individuals, an evidence

supported by the observed peak shifts between areas with different transmission levels (Appleby *et al.* 2012; Mitchell *et al.* 2011; Mutapi *et al.* 2007).

The association between levels of IgA, IgE and IgG subclasses responses against different life-stage antigens and resistance to re-infection has been studied in *S. japonicum*, *S.mansoni* and *S. haematobium* (Jiz *et al.* 2009; Langley *et al.* 1994; Mutapi *et al.* 2008; Naus *et al.* 1998; Ndhlovu *et al.* 1996). Immuno-epidemiological studies in human schistosomiasis (more in section 1.9.5) have demonstrated characteristic age-antibody profiles similar to the peak shifts observed with infection intensity (Figure 1.4). These age-antibody profiles further support the evidence of an involvement of protective immunity against infection/re-infection (Mitchell *et al.* 2012; Mutapi *et al.* 2007).

Since the focus of this thesis is on paediatric schistosomiasis, the study population was restricted to children aged between 1–10 years old, who are yet to develop acquired protective immunity. Thus, antibody responses associated with resistance to infection are not investigated in this thesis. A detailed discussion on the subject can be found in Mitchell (2010).

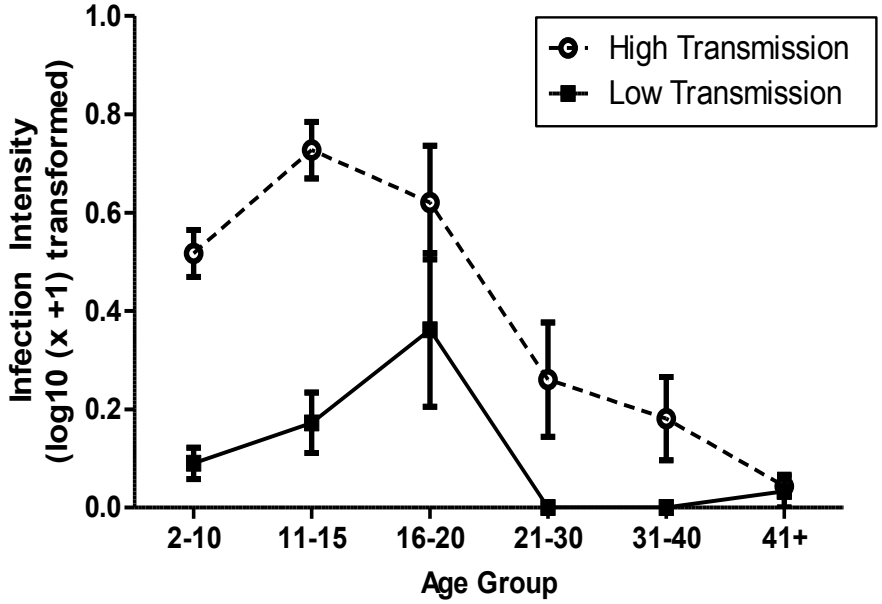


Figure 1.3: Age-infection intensity profiles indicating the peak shift. The plot shows the age-intensity of *S. haematobium* infection between populations of high and low transmission areas. The peak infection intensity in the area of high transmission (dashed line) occurred earlier in age compared to that of low transmission area (solid line). Adapted from Appleby *et al.* (2012).

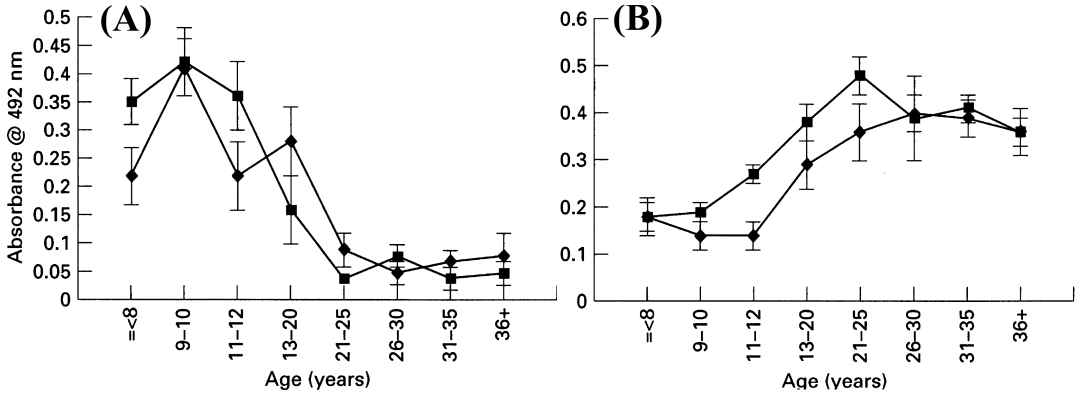


Figure 1.4: Age-related changes in the antibody profiles associated with resistance to infection. (A) IgA and (B) IgG1 against *S. haematobium* soluble egg antigen (SEA). Diamonds indicate areas of low infection and squares show high infection areas. Reproduced from Mutapi *et al.* (1997).

1.9.4 Mechanisms of immune evasion

Schistosomes have evolved mechanisms for evading the immunological defences of the host, thereby enabling them to survive for many years inside the body. The mechanisms involved in the immune evasion include mimicking of the host antigen presentation, modulation of the host immune response, and inhibition of the immune response by the parasite. The quick transformation by the cercariae to schitosomula enables them to easily migrate through the veins, avoiding the local inflammatory reactions as a result of innate immune responses against the proteins of the shed-off cercarial layer (Forrester & Pearce 2006; Pearce & MacDonald 2002). Research indicates that the schistosomula are able to manipulate the initiation of the immune responses, acquiring additional ability to resist immune attack and evade recognition by antigen-specific components of the immune response as they develop and migrate to the lungs (Angeli *et al.* 2001; Hervé *et al.* 2003). Despite being in constant contact with the immune system of the infected host, adult worms are able to survive for extended periods in the blood veins of the host. The large size of the mature adult worms makes it difficult for the host immune system to eliminate them by phagocytosis. The tegument is made of a lipid bilayer that enables the schistosomes to resist corrosion due to immune responses (Loker & Mkoji 2005; Skelly 2005). In addition, adult worms seem to incorporate host proteins into their outer surface structure to disguise themselves as host and reduce their antigenicity. This reduced surface antigenicity gives the schistosomes the unusual ability to escape immune recognition (Pearce 2005*a*;

Wakelin 1996). The parasite also makes use of some of the cytokines released during the infection for its survival. Several cytokines have been implicated as important for the growth, maturation and survival of the schistosomes. The cytokines IL-5, IL-6 and IL-7 have been associated with schistosome development and migration (Forrester & Pearce 2006). IL-10, an important cytokine in the regulation of pro-inflammatory responses is suggested to be important for evasion of the immune response by the schistosomes and TNF-α is important for egg production (Pearce & MacDonald 2002). The ability of the parasite to withstand attack by the host immune system further complicates the task of controlling and eradicating schistosomiasis in affected regions, highlighting the urgent need for an effective vaccine.

1.9.5 Immunoepidemiology of schistosomiasis

In light of the host immune defenses discussed above, not every exposure to the parasite will lead to the successful development of a mature infection. Susceptibility of the host to schistosome infections may depend on various factors as suggested by several immunological studies. Field studies on the epidemiology of schistosomiasis have shown that in endemic areas some individuals are predisposed to heavier infections than others (Kabatereine *et al.* 2004), a factor attributed to genetic variations in the population (Isnard *et al.* 2010), also reviewed by (Quinnell 2003). It has also been suggested in the literature that biological factors such as hormonal changes (e.g. due to human development, pregnancy or lactation) which influence the responsiveness of the immune system may also affect susceptibility to schistosome infections (Fulford *et al.* 1998; Wakelin 1996). Other factors identified as potential confounders to the immunoepidemiology of schistosomiasis include host age, sex, nutritional status, and history of previous exposure to infection (Milner *et al.* 2010; Mutapi *et al.* 2011; Sacko *et al.* 2011; Woolhouse *et al.* 2000). The level of exposure to parasite antigens in a population can also vary with patterns of exposure to infective waters, which may also be dependent on sex and age (Chandiwana & Woolhouse 1991; Pinot de Moira *et al.* 2010). The study of epidemiological patterns is important in understanding the pathology that occurs as a result of immune-mediated responses to schistosome infections, as well as help identify correlates of current and future morbidity that can be targeted for effective control by facilitating timely treatment of affected individuals.

The nutritional status of an individual contributes towards development of a functioning immune system. Poor nutrition can result in immune suppression, hence increased susceptibility to infections and severity of disease (Chandra 1997). In paediatric schistosomiasis, malnutrition has been considered as a factor able to modify immunity, increasing the severity of schistosome-related morbidity and resulting in reduced physical growth and poor cognitive development (King *et al.* 2005; Sacko *et al.* 2011), reviewed in detail by McGarvey (2000).

Age-related changes in antibody responses have been reported in several studies, indicative of differences in levels of exposure, susceptibility, or resistance to infection at population level (Hagan 1992; Mutapi *et al.* 1997; Woolhouse *et al.* 2000). IgM antibodies directed against the schistosome egg antigens have been shown to increase with age, reflecting differences in cumulative exposure to schistosome infection (Milner *et al.* 2010; Mutapi *et al.* 1997; Ndhlovu *et al.* 1996). In addition, IgM is a potential useful marker of current infection as it has been shown to increase rapidly with infection prevalence (Mutapi *et al.* 1997; Woolhouse *et al.* 2000). Its utility as a serological diagnostic tool in young children is evaluated in the current thesis. An increase in the levels of IgM following treatment with praziquantel has been reported, occurring as a result of an increased circulation of schistosome antigens resulting from the death of the adult worms (Mutapi *et al.* 2003). Patterns described in field studies have shown a convex shaped IgG1 and IgG4 response with age similar to the age-infection intensity profiles and these observations have been associated with susceptibility to infection (Naus *et al.* 1998; van Dam *et al.* 1996). In addition, levels of IgG2 and IgG4 subclasses have been reported to follow a similar pattern with IgM, suggesting the potential use of IgG subclasses as markers of current or past exposure to the parasite (Butterworth 1998; Zhang & Mutapi 2006). The increase in IgG3 levels with age has been associated with the development of protective immunity (Mutapi *et al.* 2006). Levels of IgE against the adult worm have been shown to increase progressively with age, but are negatively associated with reinfection intensity (Hagan 1992; Mutapi *et al.* 1997), indicative of the involvement of IgE in resistance to infection/re-infection (Jiz *et al.* 2009). Furthermore, IgA antibody responses to the protective recombinant *S. mansoni* antigen has been shown to increase with age, suggesting that IgA antibodies might be involved in the protective immune response against schistosomiasis in addition to IgE (Grzych *et al.* 1993).

Cytokines play an important role in regulating the host immune response to infection. However, chronic exposure to the cell-mediated immune response can be damaging to the host, eventually resulting in inflammation and fibrosis (Pearce & MacDonald 2002). The study of cytokine-age profiles can be useful in understanding the rate of development of parasite-specific immune responses (Mutapi *et al.* 1997). In addition, levels of systemic cytokines which mediate pro-inflammatory and anti-inflammatory responses can be useful risk indicators of current and future schistosome-related morbidity in infected individuals (Caldas *et al.* 2008; De Souza, Robson Da Paixão *et al.* 2012). The observed age-related patterns of cytokines in the field are not well understood, with different studies reporting varying patterns. In one study conducted in a *S. haematobium* endemic population, plasma levels of IL-5 and IL-10 were shown to vary with host age irrespective of the infection status (Milner *et al.* 2010). In two different studies in *S. mansoni* and *S. haematobium* endemic areas in Uganda and Zimbabwe respectively, contrasting age-cytokine profiles were reported (Joseph *et al.* 2004*a*; Mutapi *et al.* 2007). Distinct age-related cytokine patterns were observed among infected individuals in the Zimbabwean study (Mutapi *et al.* 2007), yet the study in Uganda did not find any coherent patterns between cytokine production and age (Joseph *et al.* 2004*a*). These differing patterns may be associated with species-specific differences, variability in immune responses between the populations in addition to other factors such as host genetics (Mutapi 2001).

1.9.6 Effects of treatment on immune responses

The damaging effects of praziquantel on the adult schistosome tegument expose the worm antigens to the host immune system, resulting in susceptibility of the parasite to cellular and antibody-mediated immune attack, eventually leading to the death of the parasite (Doenhoff *et al.* 2008; Harnett 1988). Thus, the schistosomes are killed as a result of the synergistic actions of chemotherapy and host immune response (Doenhoff 1989; Harnett & Kusel 1986; Redman *et al.* 1996). Several studies have shown that treatment alters the immune response to parasites, evidenced by changes in the levels and types of antibody responses and cytokines following chemotherapy (Corrêa-Oliveira *et al.* 2000; Mutapi *et al.* 2005; Pinot de Moira *et al.* 2010). Some of these treatment-related changes have been shown to vary with age, suggestive of the development of resistance to re-infection following chemotherapy (Mutapi *et al.* 2003). In most studies, levels of

IgM have been shown to increase after chemotherapy, and this has been attributed to enhanced adult worm and egg antigen recognition by the host immune system resulting from the effects of PZQ (Mutapi *et al.* 2003; Naus *et al.* 1998; Rujeni *et al.* 2013). Studies in *S. mansoni* and *S. haematobium* have also reported an increase in IgE levels following treatment, induced by the killing of the adult worms (Caldas *et al.* 2000; Mutapi *et al.* 1998; Rujeni *et al.* 2013; Walter *et al.* 2010). In a study by Mutapi *et al.* (1998), a switch in predominantly schistosome-specific IgA antibody responses 12 weeks post-treatment to a predominantly IgG1 response was observed. In addition, the findings showed that the isotype switch occured slowly with age, suggesting a beneficial "immunizing effect" of PZQ treatment (Mutapi *et al.* 1998). Treatment-induced changes in cytokine levels have been observed in endemically exposed populations. An increase in levels of cytokines IL-4, IL-5, and IL-13 have been reported, occurring immediately or within a few weeks after antihelminthic treatment (Joseph *et al.* 2004*b*; Mduluza *et al.* 2009). In a different study, IL-10 levels (a regulatory cytokine) were shown to decrease one year after treatment in individuals endemically exposed to *S. mansoni* infection (Martins-Leite *et al.* 2008). Although the effect of chemotherapy on the immune responses to schistosomes has been widely investigated in older individuals, there are quite few immunological studies focusing on infants and preschool-aged children.

1.10 Diagnosis of schistosome-related morbidity

The damage due to chronic schistosomiasis manifests itself on several different organs of the human host including the bladder, ureters, reproductive tract, intestinal tract, liver, lungs, and kidney disorders, among many other clinical complications. The symptoms of schistosomiasis varies with species and stage of infection (section 1.5). Of all forms of human schistosomiasis, infection with *S. haematobium* is associated with the highest prevalence of clinical morbidity. One of the major objectives of control programmes is the reduction or elimination of morbidity due to schistosome infection (WHO 2010). To achieve this goal, tools are needed to accurately assess the burden of morbidity and monitor the effectiveness of interventions (Vennervald & Dunne 2006). Several different methods have been used as research tools or in control programmes to quantify schistosome-related morbidity and these include:

Urine examinations: Blood in urine (haematuria) is the classical sign of urogenital schistosomiasis, occurring as a result of *S. haematobium* eggs passing out of the body inflicting physical damage to the tissues of the urogenital tract and bladder walls (Gryseels *et al.* 2006). Haematuria in infected individuals can be either gross (visible or macrohaematuria) or microscopic (microhaematuria). Evaluation of the presence of macrohaematuria can be easily performed by visual inspection of urine specimens of infected individuals. Microhaematuria can be detected either by microscopy or rapidly using urinalysis dipstick reagent strips (Milford 2008). The results of urinalysis can be graded semi-quantitatively to indicate total absence (negative) or severity levels (e.g. +, ++, +++) of the detected microhaematuria (Sacko *et al.* 2011). In recent years, the use of dipsticks has gained wide application in large-scale field studies as they are non-evasive and do not require advanced technical skills to use (King & Bertsch 2013; Sousa-Figueiredo *et al.* 2009; Stothard 2009). In addition, proteinuria and albuminuria, also detectable semi-quantitatively by urinalysis using reagent strips have been utilized as biological markers of urinary tract pathology and as early predictors of progressive kidney disease associated with schistosomiasis (Eknoyan *et al.* 2003; Houmsou *et al.* 2013; Sousa-Figueiredo *et al.* 2009; Stothard 2009).

Questionnaires: Symptoms commonly associated with schistosome infection such as macrohaematuria, dysuria, suprapubic and/or abdominal pains usually can be easily perceived by infected individuals (Chen & Mott 1989). Thus, self-reported questionnaires can be used to assess current presence or history of schistosome-related morbidity. The use of standardized questionnaire is recommended by the WHO for rapid screening of *S. haematobium* infection and morbidity, and these have been extensively used in endemic regions (van der Werf *et al.* 2002; WHO 2002).

Physical examination: Non-intrusive clinical examination conducted by qualified medical personnel, involving abdominal palpation can be used to determine current health status and screening for early schistosome-related abnormalities (Gray *et al.* 2011; Müller *et al.* 2011). The frequency of detected clinical symptoms can be used to estimate the prevalence of schistosome-related morbidity in the population (Sacko *et al.* 2011). However, physical examination has been shown to be less sensitive (Doehring 2010), requires skilled personnel, and can be subjective and difficult to perform in non-cooperative subjects such as very young children (Doehring 2010; Pinto-Silva *et al.* 2010).

Ultrasonography: The use of ultrasonography has been shown to be effective in detecting organ-specific morbidity due to schistosomiasis (Doehring-Schwerdtfeger *et al.* 1992; King *et al.* 2003; Richter *et al.* 2000). Examples of schistosome-related morbidity detected by ultrasonography include schistosomal bladder and kidney lesions, thickened and irregular bladder walls, hydronephrosis of the kidney, and liver or spleen abnormalities (Chen & Mott 1989; Leutscher *et al.* 2000*b*; Sacko *et al.* 2011). Although a valuable tool for clinical diagnosis, the need for specialized equipment and trained personnel reduces the utility of ultrasonography for large population studies in the field (Richter *et al.* 2000).

Serological and immunological biomarkers: Several different molecular biomarkers and immune correlates can be measured in the blood and used as indirect indicators of infection or underlying morbidity. Examples of biomarkers whose concentration levels can be quantified in serum are introduced in this section. Their relevance as potential indicators of schistosome-related morbidity will be further clarified in respective subsequent chapters. Serum biomarkers such as YKL-40 or chitinase 3-like-1 protein (CHI3L1), C-reactive protein (CRP), ferritin, resistin, secretory leukocyte protease inhibitor (SLPI) have been evaluated and associated with disease conditions including liver cirrhosis, asthma, viral hepatitis, HIV, pulmonary tuberculosis and other inflammatory conditions (George *et al.* 2014; Jang *et al.* 2015; Lin *et al.* 2004; Olveda *et al.* 2014; Ribeiro 1997). CHI3L1 is a glycoprotein that is up-regulated in a variety of human conditions including cancers. It is expressed by many different cells and has been found to play a role in Th2 pro-inflammatory responses (Kzhyshkowska *et al.* 2007; Lee *et al.* 2011). Elevated serum levels of CHI3L1 are associated with chronic inflammation and tissue remodelling (Bonneh-Barkay *et al.* 2010; Mizoguchi 2006). In addition, reports have indicated that increased levels of CHI3L1 correlate with the severity of disease (Coffman 2008). In intestinal schistosomiasis, CHI3L1 has been identified as a marker of liver fibrosis (Olveda *et al.* 2014; Zheng *et al.* 2005), and more recently it has been shown to be associated with presence of haematuria in individuals infected with *S. haematobium* (Appleby *et al.* 2012).

CRP is a known acute phase protein produced by the liver in response to inflammation (Macy *et al.* 1997), and thus in schistosomiasis it can be utilized as non-specific early predictor of morbidity due to chronic infection (Coutinho *et al.* 2006; Macy *et al.* 1997; Ribeiro 1997). High concentration of serum ferritin results as a leakage product of damaged cells (Kell & Pretorius 2014), and has

been associated with iron deficiency anaemia associated with different disease conditions (Beasley *et al.* 1999; Bhargava *et al.* 2003; Hall *et al.* 2008; Suominen *et al.* 1998). Ferritin has been identified in other studies as a potential biomarker of inflammation in helminth infections in humans (Lee *et al.* 2002; Mcsharry *et al.* 1999). Other serological markers of inflammation that have been previously shown to be important in the study of a variety of infections, reflecting disease activity and/or severity include resistin (Nair *et al.* 2006; Silswal *et al.* 2005) and SLPI (Gipson *et al.* 1999; Jin *et al.* 1997; Lin *et al.* 2004). However, their validity as potential markers of schistosome-related morbidity is still yet to be elucidated.

Identification of serological correlates of current and future schistosome-related morbidity may prove to be important tools for the assessment of treatment interventions and hence aid in the design of improved future control programmes. However, currently, research on the performance of such serological biomarkers in the evaluation of morbidity due to schistosomiasis in children is still limited.

1.11 Quantifying immune response markers

Circulating levels of antibodies, cytokines and inflammatory protein biomarkers can be quantified using laboratory based techniques such as the enzyme-linked immunosorbent assay (ELISA). The basics of an ELISA makes use of the immunological concept of antibodies produced in response to pathogens binding to their antigen targets with great specificity, which allows the detection of analytes produced by the immune system such as proteins, peptides or antibodies in serum (Crowther 2001; Gan & Patel 2013). There are three main methods of ELISAs, namely; the indirect, direct, and sandwich ELISA, as illustrated in Figure 1.5. These three groups of assay can be used to form the basis of an additional assay method, the competition ELISAs.

The typical steps involved in an ELISA protocol to detect a pathogen-specific antigen are shown in Figure 1.6. These can be modified according to the type of the ELISA method performed (Gan & Patel 2013). As shown in Figure 1.6, ELISAs are performed in micro-titer plates (e.g. 96-well polystyrene plates), which can bind antibodies and proteins. This micro-plate surface binds molecules through hydrogen bond formation which immobilizes the reactants of the ELISA, making it possible to washout the unbound material during the assay. The whole surface of the plates must be blocked with non-specific protein solution (e.g. bovine serum

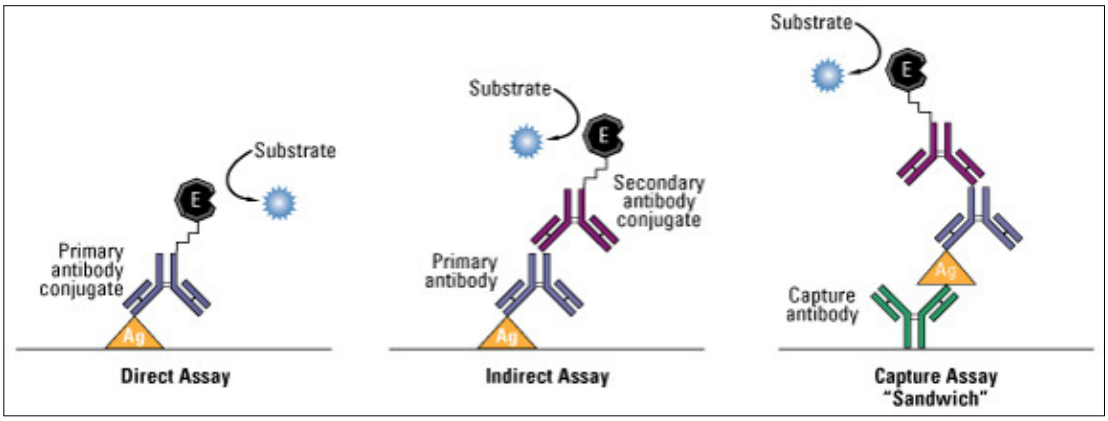


Figure 1.5: Schematic representation of the different types of ELISAs. Adapted from online resource: http://www.piercenet.com/method/overview-elisa [Accessed: 10/06/2014], with kind permission of the copyrights holder.

albumin) to ensure the specific binding of the components added in the subsequent steps. The enzyme attached to the secondary antibody has a negligible effect on the binding properties of the antibody and following the addition of a substrate for this enzyme there is a change in colour upon reaction with the enzyme.

A spectrophotometer is used to give quantitative values for colour strength, read in optical densities at specific wavelengths for the distinctive colours obtained with particular enzyme systems. The enzyme acts as an amplifier, even if only few enzyme-linked antibodies remain bound, the enzyme molecules will produce many signal molecules (Crowther 2001; Gan & Patel 2013; Walker 1995). The enzyme can go on producing colour indefinitely, hence readings should be taken within a limited time. However, an additional step is performed by adding reagents to stop the reaction of the enzyme with the substrate.

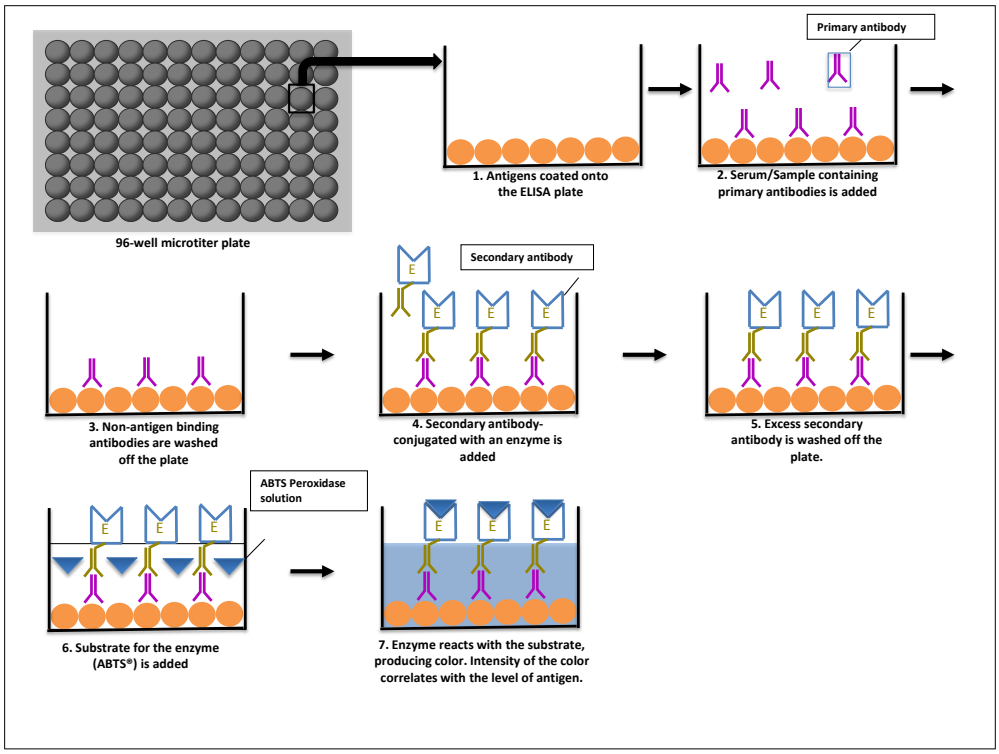


Figure 1.6: The basic steps for performing an enzyme-linked immunosorbent assay (ELISA) procedure. Reproduced from (Gan & Patel 2013) with permission from the licensed content publisher.

1.12 Outline of the thesis

Preschool-aged children have been neglected in terms of research and control of schistosomiasis. In light of the recent WHO recommendation to include these children in control programmes (WHO 2011*a*), it is necessary that revisions are made to the current control strategies. This thesis will address the present gap in the knowledge about factors associated with infection and morbidity in preschool-aged children to better inform the design and implementation of improved programmes aimed at controlling morbidity associated with schistosomiasis in this age group. An overview of each of the chapters within this thesis now follows.

In **Chapter 2**, the aims and design of the study are outlined. The methods applied within the thesis chapters are also described. This chapter will also provide a broad view of the choice of several statistical methodologies used in this thesis. Basic notations, general definitions and fundamental concepts to be used consistently throughout the thesis are also outlined in this chapter.

The WHO guidelines for schistosomiasis control stipulate that infection prevalence must be determined prior to the implementation of control programmes. Furthermore, the strategies adopted for intervention will depend on the predetermined infection levels. In **Chapter 3**, I compare the performance of the serological and dipstick microhaematuria infection diagnostic techniques to the standard parasitology (egg counts) method for detecting *S. haematobium* infection in preschool-aged children. The implications of the infection levels determined using the different diagnostic methods for control programmes in this age group are also investigated in this chapter.

In **Chapter 4**, I compare the utility of available point-of-care (POC) morbidity diagnostic tools in preschool *vs.* primary school-aged children (6–10 years old) and determine markers which can be used in the field to identify and quantify *S. haematobium*-related morbidity. This is the first study to validate several indicators of schistosome-related morbidity in preschool-aged children in a single population, with findings of clinical and public health importance.

Comparative assessment of health benefits of single *vs.* repeated PZQ treatment on schistosome-related morbidity in preschool and primary school-aged is the subject of **Chapter 5**. To my knowledge, this is one of the few longitudinal studies investigating the effects of antihelminthic treatment over a period of two years (biennial *vs.* annual treatment) on schistosome-related morbidity in preschool-aged children compared to children of primary school age. As such, it provides an initial operational recommendation for future studies on the subject.

Chapter 6 aims to validate the potential of several serological biomarkers of inflammation as indicators of current and future schistosome-related morbidity in children aged 1–10 years naturally exposed to *S. haematobium* infection. In addition, the effect of PZQ treatment on the circulating levels of validated correlates of schistosome-related morbidity one year following chemotherapy is investigated in this chapter.

Chapter 7 provides a general discussion of my findings, how these relate to the existing literature, and recommendations for future control programmes targeting children aged 5 years and below. Proposals for future research in the field are also given.

Details that are excluded from the main text and publications are provided in the **Appendix** sections of this thesis. Publications related to the work presented in this thesis are also included at the end of this thesis.

Chapter 2

Study Aims, Design and Methods

2.1 Introduction

Schistosomiasis can be a life-long chronic disease causing a wide range of morbidity if left untreated, impacting on the general health and well-being of infected individuals (Bergquist 1992; Caldas *et al.* 2008; King & Dangerfield-Cha 2008; Müller *et al.* 2011). Chronic infection in children can result in stunted growth, diminished physical fitness, and impaired memory and cognition (Colley *et al.* 2014; Gryseels *et al.* 2006). In recent years, there has been a growing number of studies reporting high schistosome infection prevalence in children aged 5 years and below (Garba *et al.* 2010; Mutapi *et al.* 2011; Sousa-Figueiredo *et al.* 2008; Stothard *et al.* 2011). However, the burden of disease still remains poorly understood, and the performance of the currently available tools for detecting infection and morbidity have not yet been systematically evaluated in this age group.

The study design outlined here uses quantitative analysis of several factors measured in the field and in the laboratory associated with *S. haematobium* exposure, infection and morbidity in children aged 1–5 years, previously neglected in terms of research and schistosome control. The findings are compared with those observed in children aged between 6–10 years old who are the current targets of the schistosome control programmes. In this chapter, I describe the aims, study design, and statistical methods applied to answer specific research questions. The participants selection criteria and epidemiological features of the study cohorts are also outlined. The overall objective of this project is to determine factors associated with schistosome infection and related morbidity in children aged 1–

5 years, and evaluate new ways of reducing the current and future burden of urogenital schistosomiasis in preschool children using the antihelminthic drug praziquantel.

2.2 Study aims

The specific aims of my research project were as follows:

1. Compare the levels of *S. haematobium* infection prevalence determined using parasitological egg enumeration in urine to infection detected by serological testing for parasite-specific IgM antibody responses against schistosome egg antigens in children from an endemic area. Infection levels in 1–5 years *vs.* 6–10 years old children and their implications for control programmes were investigated (Chapter 3).

2. Compare the performance of available point-of-care (POC) morbidity diagnostic tools in children aged 1–5 years *vs.* 6–10 years old children, and determine markers which can be used in the field to identify and quantify schistosome-related morbidity (Chapter 4).

3. Relate serological inflammatory biomarkers to schistosome infection in children aged 1–10 years. The relationship of these serological markers with POC schistosome-related morbidity markers was also investigated (Chapter 5).

4. Determine if PZQ treatment improves the current health of children aged 1–5 years old by assessing schistosome-related morbidity detected by the evaluated POC diagnostic tools and changes in serological markers of morbidity. Changes in these indicators were compared with those observed in children aged 6–10 years old to determine if treatment affects the two age groups differently (Chapter 5 and 6).

5. Compare the effects of single *vs.* double PZQ treatment on the levels of validated POC markers of schistosome-related morbidity in children aged 1–5 years. The effects of the treatment regimens on these indicators were compared between the 1–5 years and 6–10 years old children to determine if the effects of repeated PZQ treatment differed by age group (Chapter 5).

2.3 Field study

2.3.1 Ethical approval and consent

The study received ethical and institutional approval from the Medical Research Council of Zimbabwe and the University of Zimbabwe (UZ), respectively. Permission to conduct the work in this province was obtained from the Provincial Medical Director through the District Educational Officer and Heads of schools in the study area. Project aims and procedures were fully explained to the community, primary-school aged children, teachers and parents/guardians in local language, Shona. Written informed consent was obtained from parents/guardians prior to enrolment of children into the study. The children were recruited into the study on voluntary basis and were free to withdraw at any time with no further obligation.

2.3.2 Study area

The study was undertaken in the Murewa district, in the north-east of Zimbabwe (31°90′E; 17°63′S) where *S. haematobium* is endemic. The study area lacks sufficient safe water and sanitation facilities (Mutapi *et al.* 2011; Nausch *et al.* 2012). Older children get exposed to infective water actively whilst playing or swimming, washing, and performing domestic chores (Imai *et al.* 2011; Mutapi *et al.* 2011). The very young children get exposed whilst accompanying adults to water sources through playing or being bathed in infected water, as confirmed by questionnaire responses of their parents/guardians (Mutapi *et al.* 2011). The area has low transmission of *S. mansoni* and soil-transmitted helminths (hookworms, *Ascaris lumbicoides*, *Trichuris trichuria*) as previously reported in other studies (Midzi *et al.* 2008; Reilly *et al.* 2008). Malaria transmission in this area is largely unstable and sporadic, giving a low prevalence of co-infections between schistosome infection and *Plasmodium falciparum* (Imai *et al.* 2011; Mabaso *et al.* 2006). The specific villages (see Figure 2.1) from which the study samples were drawn had not been involved in previous studies conducted in the area nor had they previously received antihelminthic treatment through the National Schistosomiasis Control Programme.

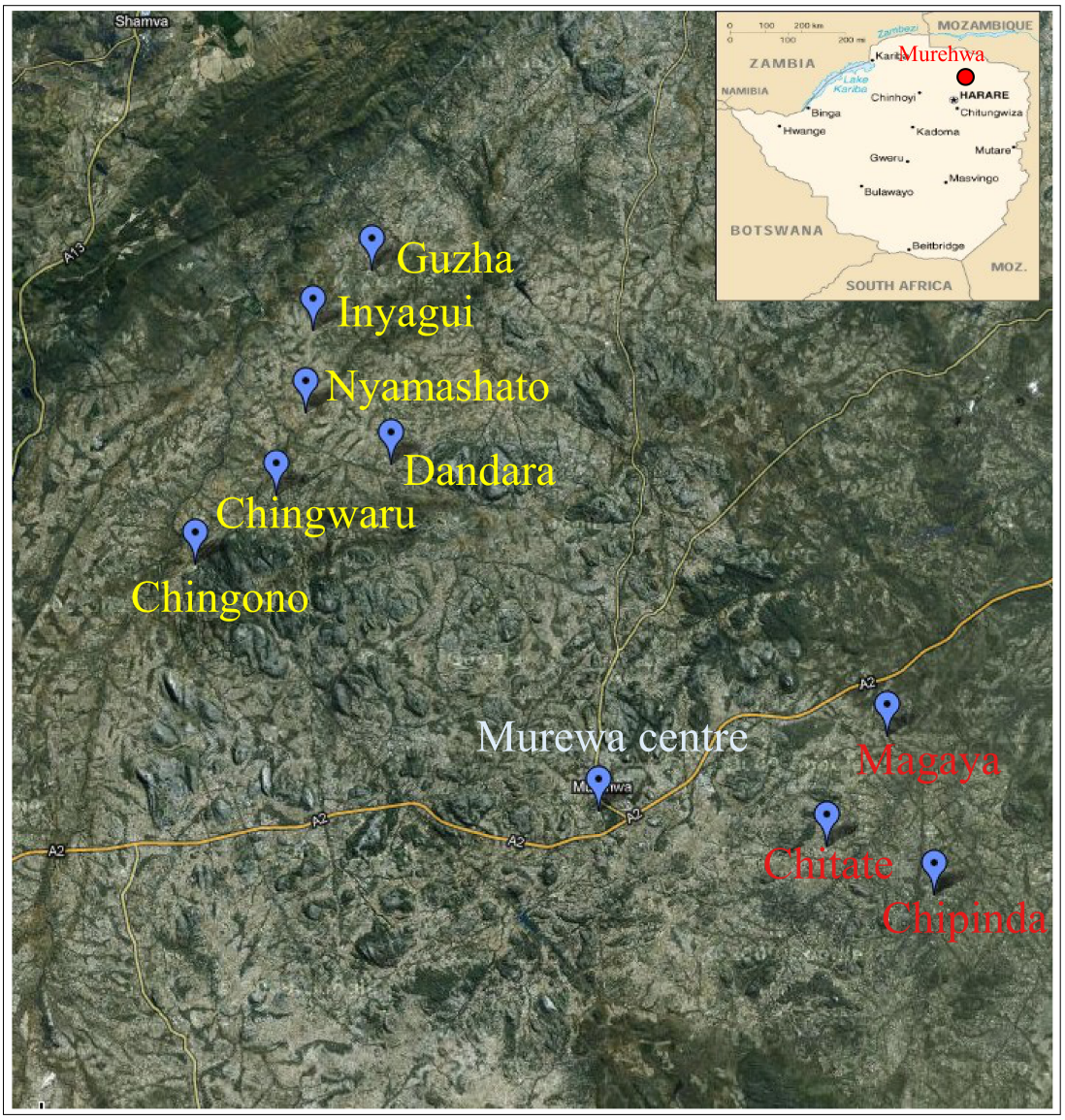


Figure 2.1: A map showing the location of the study sites in the Murewa district, North Eastern Zimbabwe. The educational recruitment centres for the present study are indicated in yellow. Neighbouring study sites involved in previous studies by my research group (Parasite Immuno-epidemiology Group) are shown in red. Image from Google maps.

2.3.3 Study design

The data considered in this thesis were obtained from field studies as part of a major project: "Health Benefits of Repeated Treatment in Paediatric Schistosomiasis" conducted in collaboration with the University of Zimbabwe (UZ), the National Institute of Health Research, Zimbabwe (NIHRZ), and the University

of Edinburgh, UK. The project consisted of cross-sectional and longitudinal designs relating infection levels, markers of schistosome-related morbidity and serological responses associated with inflammation to the number of praziquantel (PZQ) treatments children received as well as to the age of the children (ages 1–5 years and 6–10 years). For this project, I was involved in the field work that consisted of collection of urine samples for parasitology and urinalysis, data entry and coding in the field. To ensure good-quality data I facilitated double entry verification in Microsoft Excel. Prior to any statistical analyses, data was cleaned to verify and correct for any inconsistencies, re-organised and put in the format easy for storage and retrieval. Data cleaning involved investigating missing information, age verifications and conversion of these ages into standard unit (years). Blood collection for immunological assays was facilitated by experienced local nursing staff and study clinicians. Antihelminthic treatment with praziquantel was offered to all compliant children, administered by the local physician. Laboratory based work, including measurement of serological biomarkers of inflammation, antibody and plasma cytokine assays was conducted by other members of the Parasite Immuno-epidemiology Group acknowledged in this thesis (http://pig.bio.ed.ac.uk/people/edinburgh/ [Accessed 30/09/2015]).

The study design of the of the main project is a modification of the traditional treatment re-infection study designs where all participants are treated at one time point and followed up for specified time period (Figure 4.1). To ensure that the study compared re-infection rates over the same time period, compliant children received PZQ treatment 12 months into the study (antihelminthic treatment (1) in Figure 4.1 below), with an efficacy check 12 weeks later. At the end of the 24 months study period, two different groups of children having different histories of PZQ treatment were available for comparisons. Thus, Group 1 had received two treatments; at the beginning of the study (antihelminthic treatment (0)) and 12 months later (antihelminthic treatment (1)), while Group 2 will have received a single PZQ treatment (see Figure 4.1). The 12 month period between treatments was chosen to reflect an achievable target for mass chemotherapy in developing countries and in keeping with the recommendations of the 2001 World Health Assembly resolution (54.19) for biennial treatments for moderate prevalence *vs.* annual treatment in high infection areas (WHO 2002). The comparison of single biennial to two annual treatments was to investigate the health benefits of an additional treatment on infection levels and morbidity measures. Full details of

the allocation of children into different treatment groups in my study to compare the effect of single biennial *vs.* annual treatment regimens are described in chapter 5.

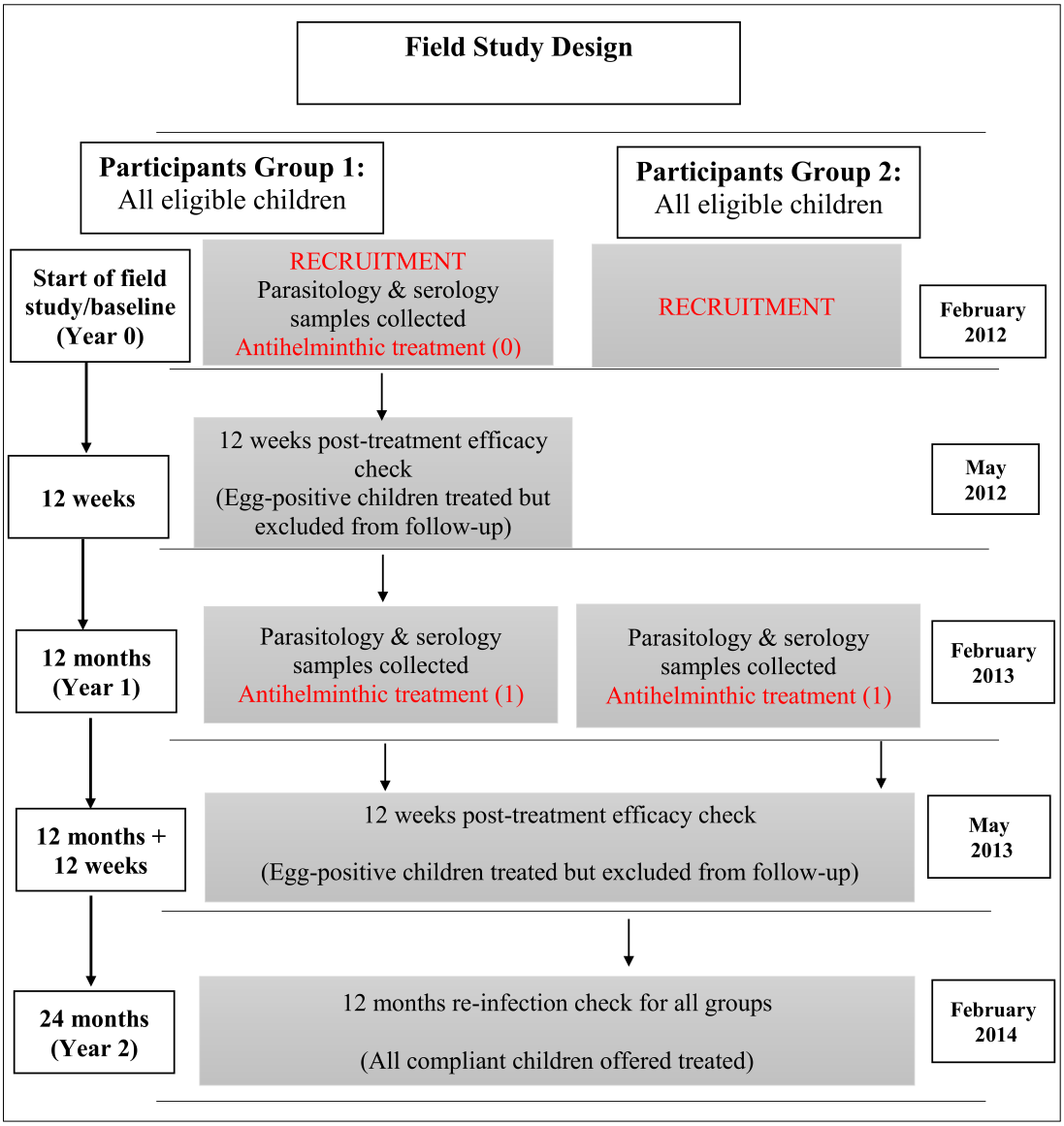


Figure 2.2: Field study design showing survey follow-up times of children with different treatment histories.

2.3.4 Study participants

Children aged 1–10 years were recruited into the study. This age group was chosen for a combination of the following scientific research, operational and welfare reasons: 1) Most of my research questions in this thesis are directed at

preschool children (aged 5 years and below) for which knowledge about the burden of schistosome-related morbidity is still scarce, evaluating existing approaches to reducing the current and future health burden of urogenital schistosomiasis in this age group using the antihelminthic drug praziquantel; 2) For children aged below 1 year, obtaining blood samples as well as the required number of parasitology samples (urine/stool samples) on designated survey days is operationally difficult; 3) Children younger than 1 year old in the study communities are not yet making independent contact with infective water and therefore have different exposure patterns to older children which can confound re-infection studies; 4) Since the study site was a high transmission area where schistosome infections peak between the ages of 9 and 10 years, by including 8–10 years old participants enabled children carrying the heaviest infections to be incorporated; 4) Children progress to secondary schools between the ages 13–14 years, therefore, in order to follow-up children for at most two years, it was essential to select children who were going to be at the same school for the study period. Eligibility criteria for inclusion in the study were as follows:

- children should have been life-long residents of the study area.
- had no prior history of antihelminthic treatment (assessed by questionnaire administered to parents/guardians for all children).
- had provided at least two urine samples for *S. haematobium* and two stool samples for soil-transmitted helminths (STHs) and *S. mansoni* parasitological examinations on consecutive days at baseline.

Children were excluded if they:

- had pre-existing known medical conditions or presented with clinical symptoms of tuberculosis, fever, or showed signs of being unhealthy upon examination by the study clinicians.
- had a recent major operation or illness as reported by parents/guardians.
- were found positive for STHs. None of the participants were positive for STHs (Hookworms, *Ascaris lumbricoides* or *Trichuris trichiura*) in all the study cohorts considered in this thesis.

For the longitudinal aspect of the study, treated children found egg-positive for *S. haematobium* at 12 weeks or 12 months + 12 weeks post-treatment efficacy checks were treated but excluded from follow-up to ensure that "true" re-infection is measured as shown in Figure 4.1.

2.3.5 Parasitology

At least two urine and two stool specimens were collected from each participant on three consecutive survey days between 10:00h and 14:00h, and processed within 2 hours of collection. For young children where it was operationally difficult to obtain the samples on the spot, urine samples were collected overnight by parents/guardians using urine collection bags (Hollister 7511 U-Bag Urine Specimen Collector, Hollister Inc., Chicago, Illinois, USA), and stool samples were collected using disposable dippers. Urine samples were examined microscopically for *S. haematobium* infection using the standard filtration method (Mott *et al.* 1982). The urine filtration equipment used consisted of a nitrocellulose mesh filter (reusable), 10 mL plastic syringe and a plastic extension tube, with the upper part of the filter attached to the mouth of the syringe. Each urine sample was thoroughly mixed and 10 mL of urine was aspirated and slowly passed through the filter on which the parasite eggs were trapped in the filter. The filter was observed under a 10X objective lens of a binoculars microscope, and number of eggs/10 mL of urine enumerated. Stool samples collected on two consecutive days were processed using the Kato-Katz method (Katz *et al.* 1972). Using this method, the stool samples were sieved to remove large particles and at least two slides per sample were prepared using standard 41.7 mg templates and stained with glycerol-malachite green to easily identify helminth eggs (this method stains stool components but does not penetrate parasite eggs). Slides were examined microscopically and egg counts per gram of stool determined for the diagnosis of *S. mansoni* and STH. Repeated parasitological examinations were performed to improve the diagnostic accuracy of the tests.

2.3.6 Blood collection

Up to 5 mL of venous blood was collected for serological assays from each participant, facilitated by experienced local nursing staff. The 5 mL blood limit was within the guidelines for children issued by the MRCZ and other institutes' research ethics committees (see Blood Sampling Guildelines accessible at: http://healthcare.partners.org/phsirb/abouthrc.htm [Accessed: 16/11/2014]). Blood samples were allowed to clot at room temperature (24°C) and stored at 4°C overnight. The clot was removed and serum extracted by centrifugation at 3000 revolutions per minute (rpm) for 10 minutes. The obtained serum samples were

stored frozen at -20°C, transported on dry ice to Edinburgh, UK and kept under storage at -80°C. Samples were thawed for the first time for the assays described below.

2.3.7 Treatment

After sample collection at baseline and at 12 months follow-up, participating compliant children (both negative and positive for schistosome infection) were offered antihelminthic treatment with PZQ at the standard oral dosage of 40 mg/kg body weight. The PZQ drug was purchased from Sigma-Aldrich (Dorset, UK), unless otherwise stated. Treatment was administered by the local physician, and for very young children, the tablets were crushed as per current recommendation by the (WHO 2011*a*). The PZQ tablets were swallowed under close supervision by study clinicians and parents/guardian with squash juice to reduce their bitter taste and a slice of bread to reduce the side effects of PZQ (Mutapi *et al.* 2011; Sousa-Figueiredo *et al.* 2010).

2.3.8 Urinalysis

The urinalysis method was used to detect current point-of-care (POC) schistosome-related morbidity markers. Urine samples collected on the first day of the survey were examined for the presence of visible haematuria, one of the early signs of urogenital schistosome-related morbidity (Gryseels *et al.* 2006). Uristix® reagent strips (Uripath, Plasmatec, UK) were used for the rapid determination of urobilinogen, glucose, blood, protein, nitrite, leukocyte, pH, and specific gravity in urine. Briefly, the reagent end of the test strip was dipped into fresh, well-mixed urine for 40 seconds. Upon removal, the test area was compared with a standard colour chart. The dipstick test results were calibrated following the manufacturer's guidelines and data recoded for easy referencing of the results in the field (see Table B.1 in Appendix B). In addition, a subset of urine specimens chosen at random was further analysed using the Multistix® 10SG (Bayer, UK) and the results (glucose, blood, protein, ketone, specific gravity, pH, nitrite, and leukocyte) read automatically using Siemens' CLINITEK Status®+ Analyzer (Bayer, UK). CLINITEK® Microalbumin Reagent Strips (Bayer, UK) were used to measure levels of albuminuria determined from the concentrations of urine albumin-creatinine-ratio (UACR), a proxy for urinary tract damage and an early

predictor of progressive kidney disease (Eknoyan *et al.* 2003; Levey *et al.* 2005; Stothard *et al.* 2009).

2.3.9 Questionnaires

A questionnaire constructed in English and translated to the local language (Shona) was used to assess dichotomous responses ('yes' or 'no') pertaining to self-reported blood in urine (haematuria) and painful urination (dysuria). Parents/guardians responded to the questions on behalf of preschool children (1–5 years old) whilst primary school-aged children (6–10 years) responded to the questions themselves. A copy of the questionnaire can be found in Appendix G.

2.3.10 Clinical examination

All participants underwent a non-intrusive physical clinical examination, involving abdominal palpation, conducted by study clinicians to determine current health status and schistosome-related anomalies (e.g., epigastric or abdominal pains).

2.4 Antibody assays

The sera obtained from blood samples were tested for parasite-specific IgM antibody responses directed against cercarial antigen preparation (CAP) and soluble egg antigens (SEA) using enzyme linked immunosorbent assays (ELISAs) following previously published protocol (Mutapi *et al.* 1997). The antibody levels were expressed as the mean optical density (OD) value of the duplicate assays read with an Emax microplate reader at absorbance of 405nm. Full details on the actual steps involved in the ELISAs can be found in Appendix A. IgM antibodies are produced early in an infection (Warrington *et al.* 2011) and previous studies have reported a positive association between anti-egg IgM antibody responses with schistosome infection levels (Dawson *et al.* 2013; Mutapi *et al.* 2003; Stothard *et al.* 2011). Previous studies have also shown that treatment induces changes in IgM antibody response levels, a factor associated with increased exposure of the antigens to the immune system (Mutapi *et al.* 2003; Naus *et al.* 1998). Thus, IgM antibody responses were used in this project to indicate recent exposure of children to schistosomes and for the serological diagnosis of infection only at baseline, i.e. pre-treatment (Imai *et al.* 2011; Stothard *et al.* 2011; Woolhouse *et al.* 2000).

High levels of heterogeneity of IgM antibody responses directed against soluble adult worm (SWA) have been reported in previous field studies conducted in the same region as the current study site (Mutapi *et al.* 1997; Woolhouse *et al.* 2000), hence were not considered for investigation in this study.

2.5 Cytokines

Schistosome-related immunopathology results from the inflammatory responses mounted against the eggs released by the parasite. This immunopathology is mediated by pro-inflammatory immune responses whose markers can be detected serologically (see Burke *et al.* (2009) for review). Measurement of these serological markers can be informative indicators of risk of future and current disease independent of schistosome infection intensity (Caldas *et al.* 2008; King *et al.* 2001). Therefore, in this thesis, plasma levels of systemic cytokines: IFN-γ, a Th1-associated response, IL-4, IL-5 and IL-13, markers of Th2 responses, and IL-10, a marker for regulatory responses were measured (Caldas *et al.* 2008; Pearce & MacDonald 2002). Since there is no single immunological marker for schistosome-related morbidity, I have chosen to assess a range of these cytokines that have been previously shown to mediate inflammatory environment during infection, and thus believed to play a key role in the pathogenesis of chronic schistosomiasis (Imai *et al.* 2011; Milner *et al.* 2010; Mutapi *et al.* 2007). Furthermore, I included both Th1 and Th2 type cytokines in the investigation to enhance understanding of the nature and development of early immune responses associated with disease in schistosomiasis, that still remain poorly defined in young children. The cytokines were detected and quantified using capture (sandwich) ELISAs following previously published protocol (Joseph *et al.* 2004*a*). The capture assay procedure has an extra added step to the basic ELISA, involving the capture antibody. ELISA kits to measure the systemic levels of the cytokines were obtained from BD Biosciences (San Diego, CA, USA).

2.6 Serological inflammatory biomarkers

Five biomarkers of inflammation indicative of the immunopathological responses were considered in this project and these included; C-reactive protein (CRP), ferritin, chitinase 3-like-1 protein (CHI3L1), resistin, and secretory leukocyte

protease inhibitor (SLPI). Some of these serological markers considered have been shown in previous studies to be important inflammatory markers in helminths (Appleby *et al.* 2012; Coutinho *et al.* 2006; Mcsharry *et al.* 1999). Serum concentration levels of these markers were quantified using the capture ELISA method, following the same principle protocol. Serum levels of CHI3L1, CRP and resistin were quantified using ELISA kit from R&D Systems (Minneapolis, USA, Catalogue number: DY2599 for CHI3L1, DY1707 for CRP, and DY1359 for resistin). Ferritin and SLPI were measured using reagents procured from the National Institute for Biological Standards and Control (Hertfordshire, UK) and Novus Biologicals (Cambridge, UK), respectively. Measurements were taken at baseline and 12 months after treatment to determine whether PZQ had an effect on the levels of these markers.

2.7 Statistical methods

In this section I present an overview of the main statistical methods considered within the thesis to test different hypotheses, with specific details explained in relevant chapters. Technical details or derivations that are excluded from the main text are provided in the appendix section.

2.7.1 Parametric and non-parametric tests

In parametric statistics the data are assumed to come from a known underlying distribution (e.g. normal distribution) and hypothesis testing is based on the assumptions made. However, in non-parametric hypothesis testing, fewer or no distributional assumptions are made, thus making it suitable for different types of data (Rosnar 2000; Sprent & Smeeton 2001).

The following parametric techniques were used for exploratory data analysis and to test for associations:

(i) Descriptive statistics, used to reveal the basic features of the data and provide summaries about the means and variability (standard deviations: SD and standard errors: SE) of the variables of interest.

(ii) The Pearson's correlation coefficient (r), to estimate the strength of association between pairs of continuous variables such as infection intensity or antibody levels with age.

(iii) The student's t-tests: (a) two-sample t-test to compare means between two independent groups (e.g. 1–5 years *vs.* 6–10 years age groups), and, (b) paired t-test to compare two measurements taken from the same individual before and after treatment.

(iv) Since the symptoms commonly associated with schistosomiasis are non-specific, I used the method of attributable fractions to quantify the proportion of morbidity attributable to *S. haematobium* infection rather than due to other causes in the population and among infected children. The attributable fractions for morbidity outcomes were estimated using adjusted prevalence estimates obtained from the logistic regression models described in section 2.7.3.

The non-parametric statistics used included:

(i). The Spearman's rank correlation (ρ), used to measure the degree of association between two variables, appropriate for discrete data or when there were reasons to question the normality of the underlying data distribution .

(ii). The Chi-square (χ^2) test, used to test for associations between independent categorical variables, and the McNemar's statistic for paired categorical data (used to compare pre-treatment *vs.* post treatment infection or morbidity prevalence levels within the same age group of children).

(iii). The Fisher's exact test, used for small sample size test for associations between categorical variables.

Several statistical regression models were also applied to investigate the research hypotheses, accounting for potential confounding factors as outlined in the next sections.

2.7.2 General Linear Models

The main inferential statistical analysis used throughout this thesis is the general linear model (GLM) technique, utilized to investigate the presence of a relationship between variables of interest, accounting for potential confounders. Since the predictor (explanatory) variables studied were measured on different scales, the GLM was chosen for its flexibility to incorporate multiple explanatory

variables (continuous or categorical) simultaneously. Thus under this framework, the analysis of variance (ANOVA) models, where all explanatory variables are categorical and the linear regression models, consisting of continuous explanatory variables were considered (Kutner *et al.* 2005). The GLM may be written as:

$$Y_i = \beta_0 + \beta_1 X_{1i} + \ldots + \beta_p X_{pi} + \varepsilon_i, \tag{2.1}$$

where Y_i, for $i = 1, \ldots, n$ is the response variable, X_j, for $j = 1, \ldots, p$ are the explanatory variables in the model, β_0 is the intercept, $\beta_1, \ldots, \beta_p$ are the regression parameters, reflecting the effect of the explanatory variables on the response, and ε_i is the error (or residuals) term. For the results of a GLM analysis to be valid, the following important conditions need to hold: 1) independence between the n individual response cases; 2) normality of the residuals (the differences between the predicted values and actual values); 3) constancy of variance of the residuals (homogeneity).

In this thesis, the main explanatory factors adjusted for in the GLMs investigating the association between the outcome (e.g., infection intensity, antibody responses, serological markers of morbidity or systematic cytokine levels) and explanatory variable of interest included: sex (M *vs.* F), age in years (or categorized into two groups: 1–5 years *vs.* 6–10 years) and village of permanent residency. Model building included all possible biologically meaningful two-way interactions between the explanatory variables. Except for hypothesized interaction terms, all insignificant interactions were dropped from the model following the step-down (or backward selection) model building procedure. Briefly, the step-down procedure begins with the model containing all candidate predictor variables. At each modelling step, the variable that is least significant (i.e., with the largest P-value) is dropped (Kutner *et al.* 2005). Using this model selection criteria, I set the significance level at which variables could be removed from the model at $P>0.20$.

Residual plots and normal probability plots were used to identify possible deviations from the underlying assumptions on the error term and where appropriate, transformation of the response variable was implemented as a remedial measure. To test if there was any relationship between the response and the explanatory variables based on the final model, the F-test of the analysis of variance was used and P-values were considered statistically significant if $P<0.05$.

2.7.3 Generalized Linear Models

Some response variables in the study were restricted to a binary scale, e.g. presence/absence of schistosome infection or related morbidity. To model these categorical responses as a function of explanatory variables, the method of generalized linear models (McCullagh & Nelder 1989), an extension of the GLM to encompass the non-normal response distributions was applied. The two main generalized linear models used for cross-sectional data were the multiple logistic regression (adjusted for sex, age and/or village of residency) and the parametric age-dependent prevalence modelling approach first proposed by Diamond & McDonald (1992) and Keiding (1991). Further details of this method are explained in chapter 3.

To determine whether morbidity prevalence decreased after treatment and to investigate the effect of single *vs.* two PZQ treatments between age groups, the method of generalized linear models with a random intercept term to account for correlation between repeated measures within each study subject, adjusting for sex and baseline infection status was used. The mixed model was formulated as follows:

$$logit(\pi_{ij}) = ln(\frac{\pi_{ij}}{1-\pi_{ij}}) = \beta_0 + \beta_1 X_{1i} + \ldots + \beta_p X_{pi} + b_i \tag{2.2}$$

where π_{ij}: is the probability of presence of morbidity at the j^{th} survey time period (pre- or post-treatment) for the i^{th} child, and the parameters, β_i's, have the same interpretation as described for the GLM. b_i: is the random effect term to account for the association between repeated measures for each child, where $b_i \sim N(0, \sigma_b^2)$ is assumed.

Two-way interaction terms between the predictor variables were included during model building, and insignificant parameters subsequently dropped using the step-down model building procedure. The general form of the mixed models to address specific study questions was the same. The generalized linear models were run using PROC GLIMMIX with a logit link function in SAS® 9.3 (SAS Institute Inc., Cary, NC, USA), and the parameter estimation was implemented using the method of penalized quasi-likelihood to account for over-dispersion (Bolker *et al.* 2009; Molenberghs & Verbeke 2005). The likelihood ratio test was used for model selection. The basic SAS code for this model is illustrated in Appendix E.1.

2.7.4 Multivariate Analysis methods

In this thesis, I used the non-metric multidimensional scaling multivariate statistical technique to identify urinary dipstick attributes associated with schistosome-related morbidity. In addition, the statistical method of principal component analysis was applied to validate different serological markers of morbidity and inflammatory responses in children infected with schistosomiasis. In what follows, I describe in detail the two methods and their implementation using appropriate software.

2.7.5 Non-Metric Multidimensional Scaling

Non-metric multidimensional scaling (NMDS), a multivariate non-parametric data reduction technique (Cox & Cox 2001) was used to identify urinary dipstick markers that contributed most to the differences in *S. haematobium*-related morbidity among a set of potential urinalysis markers (section 2.3.8). The NMDS analysis was performed using PCORD 6.08 (MjM Software, Gleneden Beach, Oregon, USA). The NMDS model runs were based on Bray-Curtis distances with no penalty on handling ties. Five hundred iterations with real data were made with 15 iterations used to evaluate model stability based on a stability criterion of 0.000001. An initial 6-dimensional NMDS was performed to find the number of axes that best represented the variation in the urinalysis data set. Using a scree plot, it was determined that a 2-dimensional NMDS with final instability of 0.0 and stress of 8.5 was adequate to account for most of the observed variability.

Pearson's correlation coefficients were used to identify the urinary dipstick morbidity markers strongly contributing to overall variability and the proportion of variability represented by each of the NMDS axes was measured using the coefficient of determination (R^2). A multi-response permutation procedure (MRPP) test was performed to assess differences in the NMDS output by sex, age-group and *S. haematobium* infection status. A total of 1, 000 permutations of the data were executed to test for significance.

Pairwise comparisons between subgroups were conducted using a combination of MRPP statistics, namely; the T-statistic: an equivalent of the Student's t-test describing the between-group separation, measure of effect size, A: indicating the chance-corrected within-group similarity and the P-value: representing the probability of having obtained as low an average within-group similarity as

actually observed (Peck 2010). Significant group differences implied that children belonging to one subgroup were more similar in terms of the morbidity markers than they would be expected if they had belonged to the other group. In addition, similarity percentage (SIMPER) analysis was conducted to further assess the individual contribution of each of the urinary dipstick morbidity markers to the overall dissimilarities between subgroups. Plots of the resultant two ordination axes by subgroups were used to reflect patterns of variability in the original multivariate dipstick morbidity responses among children as captured in the NMDS. The distance between points in the ordination space is proportional to the underlying distance measure between these points (Peck 2010).

2.7.6 Principal Components Analysis

Principal component analysis (PCA) is a multivariate data reduction technique used to explain or summarize the underlying variability of a large set of variables without loss of information through a few linear combinations of these variables, each of which explains a percentage of the total variation in the data, known as the principal components. The first component will possess maximum variability, the second principal component will be the linear combination of maximum variance that is uncorrelated with the first principal component. Thus, in general, the i^{th} principal component is the linear combination of maximum variance that is uncorrelated with all previous principal components (Johnson & Wichern 2007). In this thesis, reduction of the five serological morbidity markers (CHI3L1, CRP, Ferritin, Resistin, and SLPI) or systemic cytokines (IL-13, IL-10, IL-5, IL-4, and IFN-γ) was performed by preserving a few principal components that explain about 80 to 90% of the total variability. The results of principal components analysis were also used as inputs to regression analyses.

2.7.7 Post-hoc tests

Post-hoc comparisons were performed on the categorical explanatory variables to identify which sub-groups differed significantly each other. In all the analyses (unless otherwise stated), pairwise comparisons were adjusted for family-wise type I error using the less conservative (i.e. has low rate of false negatives) simulation-based approach (Edwards & Berry 1987). The simulation procedure provides

adjusted P-values and confidence limits that are exact up to the Monte Carlo error (Edwards & Berry 1987).

2.7.8 Calculation of diagnostic test parameters

Test parameters, sensitivity and specificity were used to evaluate the performance of the serological diagnostic test and urinalysis dipstick microhaematuria test compared to parasitological microscopic examination of eggs in urine to detect *S. haematobium* infection. The relationship between the diagnostic test results and the presence or absence of infection can be represented in a single 2×2 contingency layout shown in Table 2.1.

Table 2.1: Illustration of infection status by diagnostic test result.

		Diagnostic test result	
		Positive	*Negative*
Infection status	*Present*	a (true positives)	b (false negatives)
	Absent	c (false positives)	d (true negatives)

The sensitivity (Se) measures the proportion of infected individuals that test positive and specificity (Sp) measures the probability that the test outcome is negative in an uninfected individual, defined respectively by the conditional probabilities:

$$\begin{aligned} Se &= \mathrm{P}(\text{Test = positive}|\text{Infection = present}) \\ &= \frac{\text{True positive}}{\text{True positives + False negatives}} = \frac{a}{a+b} \end{aligned} \tag{2.3}$$

$$\begin{aligned} Sp &= \mathrm{P}(\text{Test = negative}|\text{Infection = absent}) \\ &= \frac{\text{True negatives}}{\text{True negatives + False positives}} = \frac{d}{c+d} \end{aligned} \tag{2.4}$$

Other additional parameters such as positive and negative predictive values can be used to investigate the probability that the screening test will give the correct diagnosis. Positive predictive value (PPV) is the proportion of individuals with a positive test who actually have the disease and negative predictive value

(NPV) is the proportion of those individuals with a negative test who do not have the disease. However, a limitation of these two parameters is that they are dependent on the population being tested and are directly related to the prevalence of the disease in the population, and hence their estimation is influenced by how common or rare the disease is (Parikh *et al.* 2008). Thus, in terms of schistosomiasis control programmes, the implications of PPVs and NPVs would be more useful when evaluating the cost-effectiveness of screening tests and hence were not estimated in the present thesis.

2.7.9 Bayesian modelling

In the absence of a perfect gold standard test (i.e. with 100% accuracy), the true infection status is unknown and this can bias the estimates of *Se* and specificity *Sp* as a result of misclassification (Hui & Zhou 1998). The parasitological technique, commonly used as a standard reference is less sensitive in diagnosing individuals with light infections (Bergquist *et al.* 2009).

The method of Bayesian analysis of the Hui-Walter latent model (Hui & Walter 1980) was used to estimate the diagnostic parameters *Se* and *Sp* in a single population of children in the absence of a gold standard, assuming conditional independence between the different tests. Information on priors used was elicited from published studies (Table 2.2). A Beta prior distribution was assumed for the prevalence (π), *Se*, and *Sp* in the Bayesian framework (Dendukuri & Joseph 2001). A random variable X, is assumed to follow a beta distribution with parameters (α, β), i.e., $X \sim$ beta(α; β) if it has a probability density function:

$$f(x;\alpha,\beta) = \begin{cases} \frac{1}{B(\alpha,\beta)} x^{\alpha-1}(1-x)^{\beta-1}, & 0 \leq x \leq 1, \alpha, \beta > 0 \\ 0, & \text{Otherwise} \end{cases} \tag{2.5}$$

The parameters for Beta prior distributions were calculated using BetaBuster software (written by Chung-Lung Su, available at: http://www.epi.ucdavis.edu [Accessed: 13/12/2013]). For the Bayesian analyses, three Markov chain Monte carlo (MCMC) chains were run using WINBUGS (Spiegelhalter *et al.* 2003). The first 50, 000 iterations were discarded as burn-in and the following 150, 000 iterations were kept and thinned to 100, 000 for posterior inference. Model convergence was assessed after initial burn-in using the Gelman-Rubin diagnostic

plots (Toft *et al.* 2007). For the mathematical derivation of the parameter estimates, see appendix D.1.

Table 2.2: Summary of literature sources for priors. Information was obtained on percentage (%) infection prevalence, sensitivity (Se) and specificity (Sp) used in the Bayesian modelling to evaluate diagnostic accuracy in the absence of a gold standard test.

Reference	Diagnostic	Prevalence	*Se*	*Sp*
Webster *et al.* (2009)	Parasitology		96.0	91.0
Garba *et al.* (2013)	Parasitology	50.5		
Mutapi *et al.* (2011)	Parasitology	21.0		
Sheele *et al.* (2013)	Parasitology	97.0	96.0	81.0
Kahama *et al.* (1998)	Parasitology	84.8		
	Serology	78.8	73.0	
Kinkel *et al.* (2012)	Serology		75.7	97.1
Dawson *et al.* (2013)	Parasitology		91.6	100.0
Turner *et al.* (2004)	Parasitology		84.0	
King & Bertsch (2013)	Microhaematuria		81.0	89.0
Houmsou *et al.* (2011)	Microhaematuria	32.9	64.8	89.6
Adesola *et al.* (2012)	Microhaematuria	53.9	59.3	65.8
	Parasitology	54.8		
Ayele *et al.* (2008)	Microhaematuria	43.2	80.0	91.6
	Parasitology	47.6	50.4	62.4
Bogoch *et al.* (2012)	Microhaematuria	14.2	100.0	93.0
Robinson *et al.* (2009)	Microhaematuria	7.5	97.8	58.8
Anosike *et al.* (2001)	Microhaematuria	29.5	41.0	82.0
	Parasitology	49.9		

Literature search of publications on human schistosomes in PubMed database using the key words: "schisto" AND "infect*" AND "prev*", or "schisto*" AND "human*" AND "diagnos*", or "schisto*" AND "infect*" AND "sensit*" AND "specif*", or "S. haematobium" AND "parasit*" AND "sero*" AND "antibody*".*

Prior information on dipstick microhaematuria test was solicited from literature using similar search criteria but replacing the search terms "para" or "sero*" by the search terms "dipstick" or "microhaem*" or "haematuria".*

Chapter 3

Comparing parasitological *vs.* serological determination of *S. haematobium* infection prevalence: implications for control programmes

Part of this work has been published (Wami *et al.* 2014), and a copy of the publication is included in Appendix H.

3.1 Introduction

Urogenital schistosomiasis is among the most important parasitic diseases affecting children in sub-Saharan Africa, with profound negative impacts on their health and development. Preschool-aged children (≤5 years old) have been neglected both in terms of research and control for the previously held (but now widely refuted) view that they carry insignificant schistosome infections (Stothard & Gabrielli 2007). This was further exacerbated by poor diagnosis of infection in the field (Stothard *et al.* 2011; Vennervald *et al.* 2000). Despite recent studies in Africa reporting high prevalence of schistosome infection in infants and preschool-aged children (Mutapi *et al.* 2011; Sousa-Figueiredo *et al.* 2008; Stothard *et al.* 2011), the burden of infection and morbidity in this age group still remains

poorly understood. Consequently, this oversight can have negative impacts on the effectiveness of on-going schistosome control programmes (Garba *et al.* 2010).

In accordance with the World Health Organization (WHO) guidelines, infection prevalence must be determined prior to implementation of intervention strategies to control schistosome infection (WHO 2002). To ensure that transmission levels are reduced and development of severe morbidity is alleviated, repeated mass drug administration (MDA) at regular intervals, depending on the pre-determined target population prevalence has been recommended by the WHO (WHO 2002). Thus, it is important that sensitive diagnostic tools are used in the field to detect schistosome infection with improved accuracy.

Microscopic examination of eggs in urine (parasitological diagnostic technique) is currently the widely accepted approach for detecting and quantifying *S. haematobium* infection levels in individuals or communities in need of intervention (Kinkel *et al.* 2012; Pasvol & Hoffman 2001; van der Werf & de Vlas 2004; WHO 1998). However, this method is less sensitive in detecting light infections (Bergquist *et al.* 2009; Turner *et al.* 2004), and is unable to diagnose pre-patent or single-sex infections where there is no egg production (Mutapi 2011). Such missed cases have an important role in transmission, and hence do impact on the effectiveness of control programmes (Smith & Christie 1986). Several additional methods aimed at improving the diagnosis of schistosome infection have been evaluated, although the focus has mainly been on school-aged children and adult populations. Examples of additional diagnostic techniques include antibody detection (Alarcón de Noya *et al.* 2007; Smith *et al.* 2012), urine circulating antigen detection (Ayele *et al.* 2008; Dawson *et al.* 2013; Stothard *et al.* 2006), dipstick detection of microhaematuria (Adesola *et al.* 2012; King & Bertsch 2013) and questionnaire-reported presence of haematuria (Clements *et al.* 2008; Lengeler *et al.* 2002). The elegant dipstick meta-analysis study recently published by King & Bertsch (2013) highlights the need for more investigations on different methods for detecting infection in preschool-aged children.

3.2 Aims

The aim of this chapter is to compare the levels of *S. haematobium* infection prevalence determined by the parasitological egg enumeration in urine *vs.* infection detected by the serological testing for parasite-specific IgM antibody responses in

an endemically exposed single population of children aged 1–5 years. In addition, levels of schistosome infection detected using the two diagnostic techniques are compared between the 1–5 years and 6–10 years old children to elucidate the implications of the performance of these diagnostic tests on the required interventions for preschool-aged children in control programmes. I also investigate the utility of dipstick-detected microhaematuria as an indicator of *S. haematobium* infection in addition to the parasitological technique on a subgroup of children in this study population. The findings of this chapter will be important for the planning and implementation of improved control programmes targeting preschool-aged children.

3.3 Hypotheses

To estimate and compare the accuracies of diagnostic tests, in the present chapter I sought to test the following null hypotheses:

1. The prevalence of *S. haematobium* infection determined by the parasitological and serological methods does not differ in endemically exposed children aged 1–5 years *vs.* 6–10 years old.

2. The current standard parasitological diagnostic method is not less sensitive than the serological diagnostic approach for detecting schistosome infection in children aged 1–5 years.

3.4 Materials and Methods

3.4.1 Study participants

To compare the available diagnostic tools for *S. haematobium* infection in children, a cross-sectional survey was conducted in the Murewa district study site described in chapter 2. 21 *S. mansoni* positive children were excluded in this study to reduce the possibility of cross-reactivity in serological assays (Figure 3.1). The study sample comprised of a total of 438 children considered for the final analysis (Figure 3.1), aged between 1–10 years from two villages in the study area (village 1, n=224; village 2, n=214). Since the study aimed to compare different diagnostic techniques on a single population, only participants meeting the

following inclusion criteria were considered in this current chapter: 1) had provided at least three urine samples for parasitological detection of *S. haematobium* infection, 2) had provided up to 5 mL of venous blood for serological examination, and 3) were negative for *S. mansoni* as detected by parasitological examination of at least two stool samples.

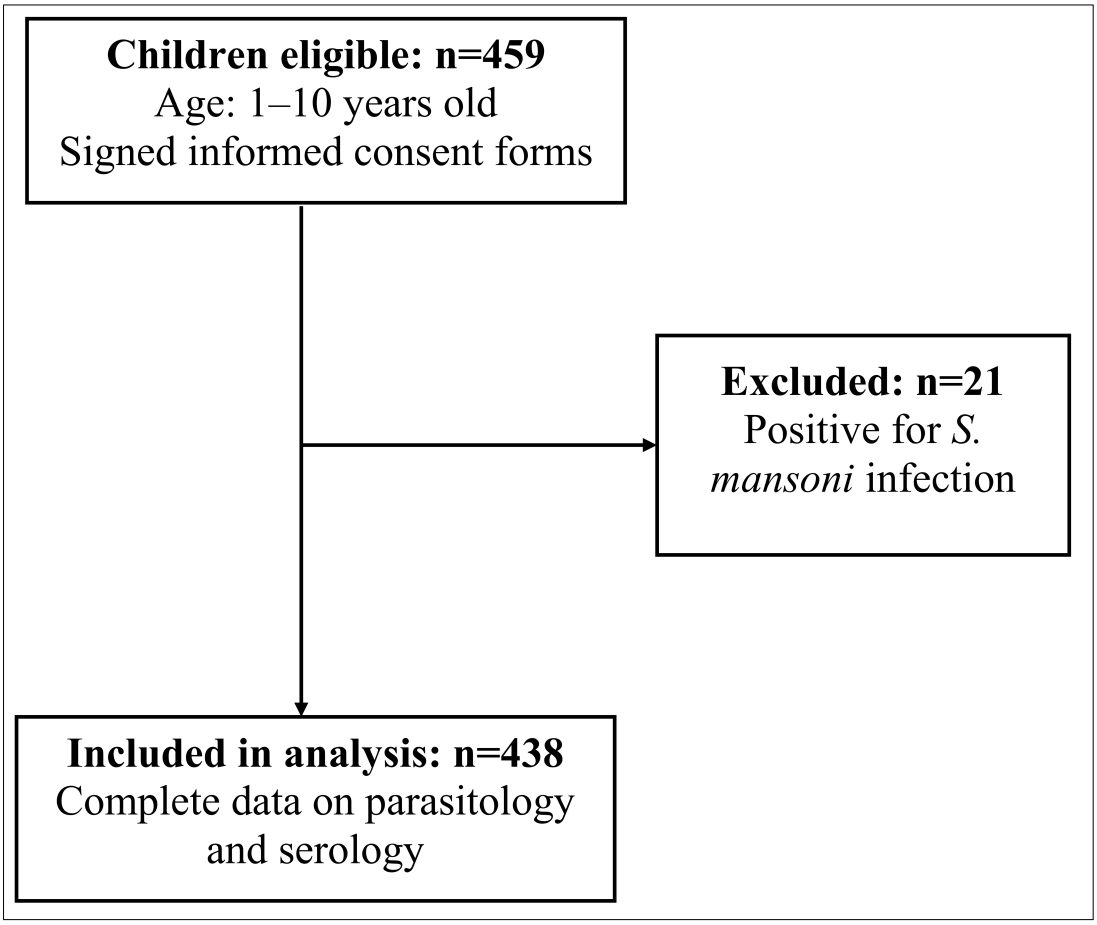


Figure 3.1: Study design flow chart indicating the number of children enrolled in the study and included in the final analysis.

3.4.2 Urine examination

Urine samples were examined microscopically for presence of *S. haematobium* eggs using the standard filtration method (Mott *et al.* 1982). Children were designated egg positive if at least one egg was detected in any of their urine samples. The infection intensity was expressed as the arithmetic mean egg counts/10 mL of the three replicate urine specimens. Examination for microhaematuria was performed

on urine samples collected on the first day of the survey using dipstick reagent strips (Uristix®, Plasmatec, UK). In this current study, due to limited resources in the field, only 190 of the initial 438 children had their urine samples examined for microhaematuria in addition to parasitological examinations.

3.4.3 Serological examination

Levels of IgM antibody responses directed against cercariae (CAP) and soluble egg (SEA) antigens were measured by the indirect ELISA method described in chapter 2. The parasite-specific antigen preparations (CAP and SEA) were sourced from the Theodor Bilharz Institute (Egypt). The ELISAs were conducted in duplicate per plate. In brief, 96-well microtiter plates (Greiner Bio-One, UK) were coated with 100 μL per well of antigen at 5 μg/mL (for CAP) and 10 μg/mL (for SEA) diluted in carbonate bicarbonate buffer at pH 9.6 per well and incubated overnight at 4°C. Plates were washed, emptied and blocked with 200 μL per well of 5% milk in phosphate buffered saline (PBS)/0.03% Tween 20 for an hour at room temperature, washed again three times and sera added in duplicate using sample dilutions 1:100. The plates were incubated for 2 hours at room temperature, washed six times and a well of substrate (ABTS peroxidase solution) added. A reaction time of 15 minutes was allowed to take place before plates were read at an absorbance of 405 nm. The results were expressed as the mean optical density (OD) value of the duplicates. Five CAP and four SEA IgM serum samples from age-matched schistosome naïve European and 13 healthy (clinically examined by experienced study clinicians) Zimbabwean donors were used as controls to determine cut-off OD values for serodiagnosis. The European samples were drawn from the Edinburgh anonymized clinical sample archive.

3.5 Data and Statistical methods

For each of the children meeting the inclusion criteria, data were available on parasitology, parasite-specific IgM antibody responses, as well as demographic information: sex (male *vs.* female), age in years, also categorized into age groups (1–5 years *vs.* 6–10 years), and village of permanent residency (village 1 *vs.* village 2). As previously highlighted, dipstick test data were only available for a subset of the study population, and as such was used for subgroup analysis.

3.5.1 Cut-off values

Across different fields of research, there is no standard rule for determining cut-offs for serological diagnosis (Baaten *et al.* 2011; Faria-Pinto *et al.* 2010; Pablo Martínez-Camblor 2011; Woolhouse *et al.* 2000), and the standardization of a specific cut-offs will be limited due to diversity in the populations being studied and the design of assay protocols (Martín-Gandul *et al.* 2013). Since the choice of cut-offs can influence the estimation of the test parameters (prevalence, sensitivity, specificity), it is thus important to make sure that the choice of these cut-offs are robust classification measures. A number of possible cut-offs can be considered and the most suitable one found. For each of the parasite-specific raw IgM antibody responses (CAP or SEA), the distribution of the raw antibody titres was initially explored by means of histograms and density curves. Using antibody data from negative controls, the cut-off values were calculated as the mean + 2 standard deviations (SD), a less sophisticated approach commonly used in literature (Imai *et al.* 2011; Kahama *et al.* 1998; Riley *et al.* 2000; Woolhouse *et al.* 2000). Antibody optical density (OD) levels greater than this cut-off value were considered to be seropositive. The robustness of the determined cut-offs to changes in sample variability was assessed as outlined in appendix C. These cut-off values were robust to changes in the mean OD variability and no bias due to individual observations were observed.

3.5.2 Determination of *S. haematobium* exposure and infection

Children were classified as exposed to the parasite if positive for CAP IgM. For each child, *S. haematobium* infection was determined using the three diagnostic methods: parasitology, serology (SEA IgM), and/or dipstick microhaematuria. Based on results of each of these diagnostic tests, presence of schistosome infection was defined as follows:

1. *Parasitology:* Egg positive, indicated by the presence of eggs in at least one urine sample.

2. *Serology:* Positive for SEA IgM antibody response, based on the OD cut-off threshold estimated from sera of negative controls.

3. *Dipstick:* Positive test for microhaematuria.

3.5.3 Statistical analyses

Infection intensity was log-transformed using $\log_{10}$(egg count + 1) to meet the underlying assumptions of parametric statistical tests. To investigate whether the mean antibody levels or mean infection intensity differed significantly between the two age groups (1–5 years *vs.* 6–10 years), independent t-tests were used. The effect of sex, age group and village on the mean infection intensity or antibody levels was investigated using general linear models (GLMs). To test for differences in infection prevalence between the two age groups and compare prevalences determined by parasitology *vs.* serology or dipstick microhaematuria, Chi-square (χ^2) tests were used.

Infection prevalence based on the binary response variable (Y_i) derived from the diagnostic test results (positive=1 or negative=0) as a function of age was estimated parametrically using the method of generalized linear regression modelling (Keiding 1991). Let n be the sample size under investigation, a_i=the age of the i^{th} child (for $i = 1, \ldots, n$) and $q(a)$=the proportion of uninfected children aged a years in the study population. The prevalence, which is the probability of being infected at age a, is given by: $\pi(a) = 1 - q(a)$, and is estimated using the binary response variable Y_i as follows: $\pi(a) = P(Y_i = 1|a_i)$. The generalized linear model with a complementary log-log link was fitted to take into account the binary nature of the response variable (Mathei *et al.* 2006), and is expressed parametrically as follows:

$$\pi(a) = 1 - exp(-\alpha a^{\beta}), \tag{3.1}$$

where α is the intercept and β is the slope, i.e. the coefficient representing the effect of age on the probability of being infected with the *S. haematobium* parasite.

The parasitological technique, widely utilized as a standard reference test is known to be less sensitive, especially in diagnosing individuals with light infections (Bergquist *et al.* 2009). Thus to assess the accuracy of the serological and dipstick microhaematuria tests in the absence of a gold standard, the diagnostic parameters; sensitivity and specificity were estimated using the method of Bayesian analysis of the Hui-Walter latent model (Hui & Walter 1980) as explained in chapter 2 (see section 2.5.8). This enabled the unbiased estimation of these test parameters, accounting for uncertainty about the diagnostic test outcomes through use of prior information in the Bayesian

modelling framework (Branscum *et al.* 2005; de Clare Bronsvoort *et al.* 2010; Dendukuri & Joseph 2001; Joseph *et al.* 1995). Information on priors used was elicited from published studies (Table 2.2). Since the choice of priors may have a strong influence on the Bayesian modelling results (Lewis & Torgerson 2012), I further explored the estimates of sensitivity and specificity by making use of vague (non-informative) priors, assuming a uniform distribution, i.e. U(0,1). The results from the Bayesian analysis using vague priors were consistent with those obtained from strong priors obtained from the literature search, hence results from the latter were reported in this study. Model convergence was assessed after initial burn-in using the Gelman-Rubin diagnostic plots (see appendix D.2). The Markov chain Monte carlo (MCMC) chains in the Bayesian models were run using WINBUGS (Spiegelhalter *et al.* 2003) following the steps outlined in chapter 2 (section 2.7.9).

3.6 Results

3.6.1 Demographics

The study population consisted of 239 (54.6%) female and 199 (45.4%) male children. Within this study cohort, 97 (22.2%) children were aged 1–5 years (median=4.0 years) and 341 (77.9%) were aged 6–10 years (median=8.0 years).

3.6.2 Infection intensity and antibody response levels

The overall mean *S. haematobium* infection intensity based on egg counts was 17.40 eggs/10 mL urine (SD=71.20) and the overall mean CAP and SEA IgM antibody levels were 0.59 OD (SD=0.38) and 0.62 OD (SD=0.34) respectively. The egg count data was further log-transformed: $\log_{10}(x + 1)$ to meet the normality assumption of parametric statistical tests. The independent *t*-tests, as shown in Table 3.1, indicated that the mean infection intensity and antibody levels were significantly higher in children aged 6–10 years compared to the 1–5 years old children. A high variability in mean egg counts in urine among children was observed as indicated by the large standard deviations in both age groups (Table 3.1). Based on the mean egg counts in urine, 7.1% (n=31) of the study participants carried heavy infection intensities, and 30.4% (n=133) carried light

infections according to the WHO classification of infection intensity burden for *S. haematobium* (WHO 2002).

Table 3.1: Mean *S. haematobium* infection intensity and IgM antibody responses directed against cercariae (CAP) and schistosome egg antigens (SEA). The independent *t*-tests were used to compare means between the two age groups. For infection intensity, the comparison test was performed on the log-transformed data [$\log_{10}$(egg count + 1)].

Variable	Age group	N	Mean	SD	Range	*t*	*P-value*
Egg count	1–5 years	97	9.03	47.53	0.00–380.33	-4.49	<0.001
	6–10 years	341	19.78	76.50	0.00–1013.00		
IgM: CAP	1–5 years	97	0.42	0.36	0.00–1.39	-5.10	<0.001
	6–10 years	341	0.63	0.37	0.03–1.90		
IgM: SEA	1–5 years	97	0.46	0.31	0.01–1.27	-5.72	<0.001
	6–10 years	341	0.67	0.33	0.07–2.39		

SD=standard deviation; Range=minimum–maximum; CAP=cercarial antigen preparation; SEA=soluble egg antigen.

3.6.3 Age-infection intensity and antibody response profiles

The distribution of the mean *S. haematobium* infection intensity and parasite-specific IgM antibody responses with age are shown in Figure 3.2. Infection intensity increased significantly with age (r=0.18; P <0.001). The IgM antibody levels against schistosome antigens showed similar patterns and there was also a significant positive correlation with age for both CAP (r=0.28; P <0.001) and SEA (r=0.31; P <0.001). The highest infection and antibody levels were observed between the ages of 8 and 10 years (Figure 3.2). There were no significant differences in the mean infection intensity (F=2.11; P=0.147), CAP (F=0.03; P=0.865), or SEA IgM (F=1.51; P=0.220) by sex (M *vs.* F), adjusting for the effects of age and village of permanent residency in the GLMs.

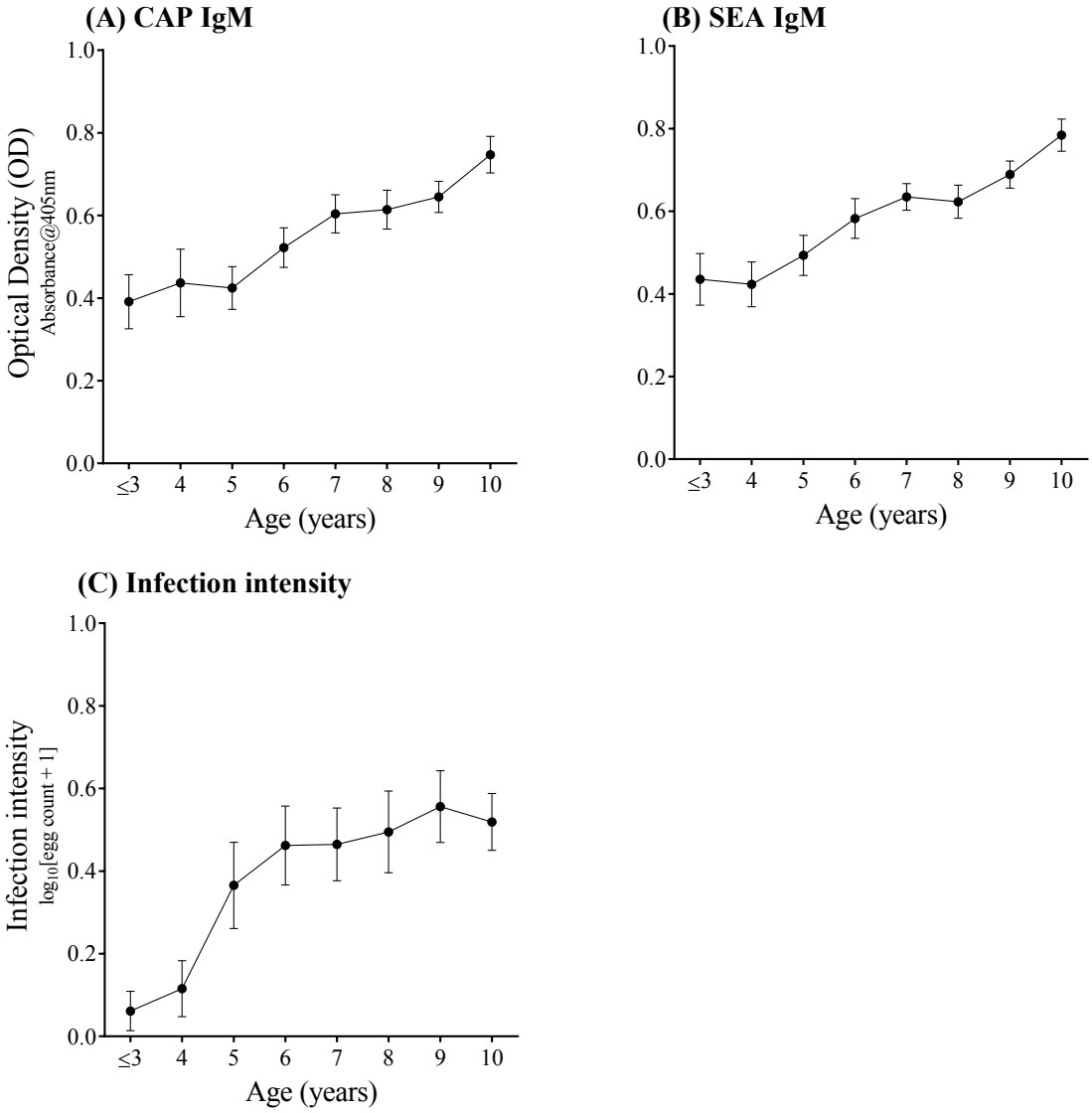


Figure 3.2: Age-infection intensity and IgM antibody response profiles. Error bars indicate the standard error of the mean.

3.6.4 Levels of exposure to schistosomes

The level of exposure to *S. haematobium* among the children was 70.6% (95% CI: 66.3–74.8%) as quantified by positive IgM antibody responses against cercarial antigens (CAP) shown in Table 3.2. In addition, a high proportion of children in both age groups showed evidence of exposure to schistosomes; 1–5 years: 48.5% (95% CI: 38.3–58.6%), and 6–10 years: 76.8% (95% CI: 72.3–81.3%). The level

of exposure to schistosomes did not differ by village, allowing for sex and age-related differences between the children (Wald χ^2=2.13; P=0.144). Furthermore, 19 schistosome egg-positive children were classified as negative for CAP IgM (Table 3.2), implying a sensitivity of 88.4% (95% CI: 83.5–93.3%) for CAP IgM as a marker of exposure to schistosomes in comparison to the parasitological diagnostic technique.

Table 3.2: Parasitological and serological diagnostic test results by age group. Levels of exposure to *S. haematobium* was defined as positive IgM antibody responses against cercarial antigens (CAP), infection was determined by positive SEA IgM for the serological diagnostic method, and by the presence of at least one egg in urine for parasitological diagnosis.

			1–5 years	6–10 years	Overall
Parasitology	**CAP**	**SEA**	**n (%)**	**n (%)**	**n (%)**
–	–	–	45 (46.4)	46 (13.5)	91 (20.8)
+	–	–	1 (1.0)	13 (3.8)	14 (3.2)
–	+	–	6 (6.2)	12 (3.5)	18 (4.1)
+	+	–	0 (0.0)	2 (0.6)	2 (0.5)
–	–	+	3 (3.1)	16 (4.7)	19 (4.3)
–	+	+	25 (25.8)	121 (35.5)	146 (33.3)
+	–	+	1 (1.0)	4 (1.2)	5 (1.1)
+	+	+	16 (16.5)	127 (37.2)	143 (32.6)
Total (N)			**97**	**341**	**438**

(+) Positive combinations of parasitological and serological diagnostic results indicating exposure or infection.

3.6.5 Schistosome infection prevalence

The overall infection prevalences based on parasitology and serology (SEA IgM) diagnostic tests were 37.4% (95% CI: 33.0–42.0%) and 71.5% (95% CI: 67.2–75.7%) respectively, and these differences were significant (χ^2=102.12; P <0.001). In addition, results of comparisons by age group revealed that infection prevalence was significantly higher in children aged 6–10 years compared with prevalence in children aged 1–5 years old, and this was true for both diagnostic tests as shown

in Figure 3.3. However, no significant differences in apparent prevalence between male and female children were observed (parasitology: χ^2=0.79, P=0.374; and serology: χ^2=0.15, P=0.703).

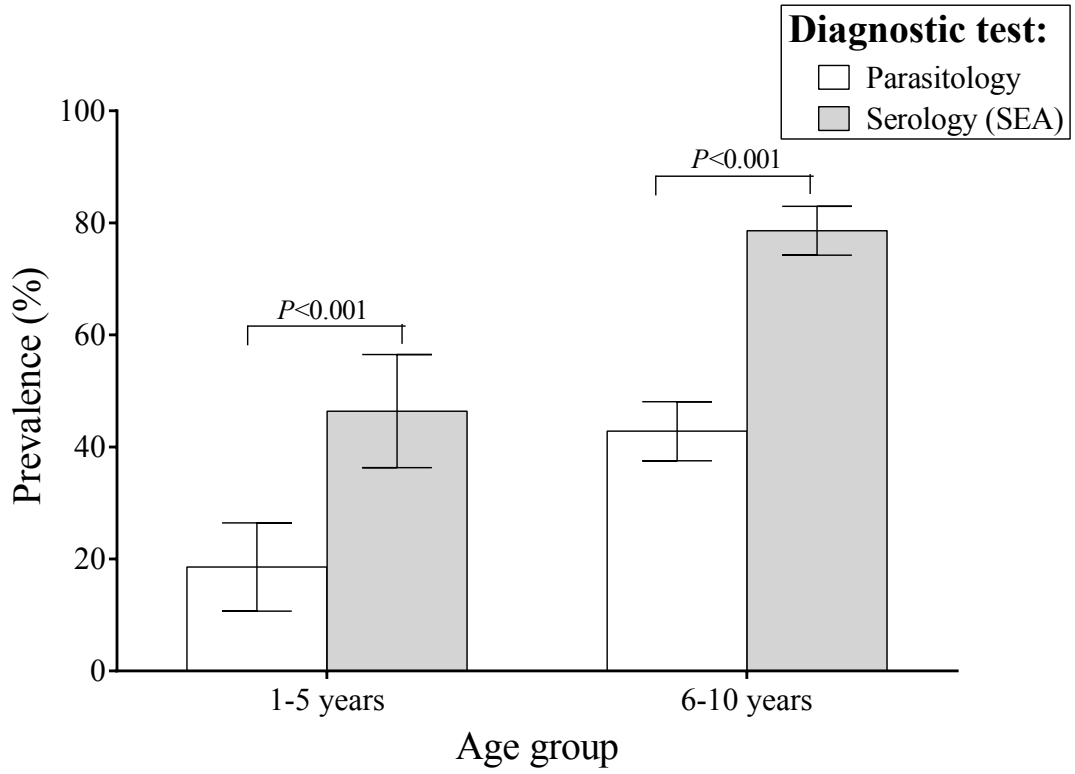


Figure 3.3: Prevalence of *S. haematobium* infection determined using parasitological and serological diagnostic methods. Schistosome specific IgM antibody levels against egg antigens (SEA) were used for serological diagnosis of infection. The error bars indicate 95% confidence intervals. The P-values are from the χ^2-test for differences in prevalence between the two diagnostic methods by age group.

3.6.6 Age-dependent infection prevalence profiles

The estimated regression coefficients and standard errors from results of the generalized linear model described earlier to determine the age-dependent infection prevalence based on parasitological *vs.* serological data were as follows: intercept, α : 0.04 (SE=0.02) *vs.* 0.12 (SE=0.05) and slope, β : 1.23 (SE=0.27) *vs.* 1.25 (SE=0.19). These parameters were used to describe the curve represented in Figure 3.4.

Infection prevalence increased with age in a similar pattern for both diagnostic methods, however the rate of increase for the serology test was higher compared to that of the parasitological technique as illustrated in Figure 3.4. In addition, the overall age-dependent infection prevalence determined using the serological technique was higher compared to the prevalence based on parasitology and this discrepancy increased with age (Figure 3.4). Furthermore, infection levels in children aged 5 years and above determined by serology belonged to the high prevalence WHO category (prevalence ≥50%) compared to the moderate (10%≤ prevalence ≤50%) category implied by the parasitological diagnostic method.

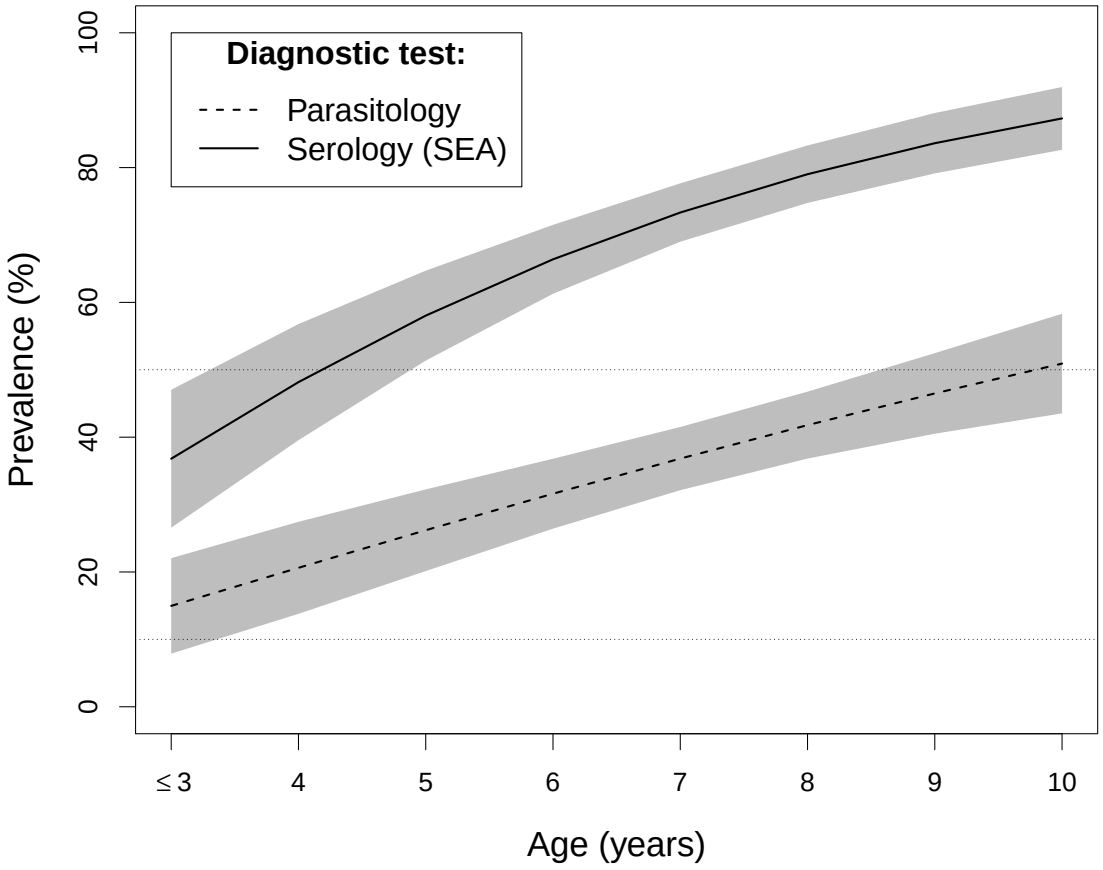


Figure 3.4: Predicted age-related *S. haematobium* infection prevalence profiles derived from parasitological (dashed line) and serological (solid line) diagnostic tests. The error bands indicate the 95% confidence intervals. The horizontal dashed lines indicate the moderate (10%) and high (50%) infection-risk cut-offs for treatment control regimens as defined by the World Health Organization.

3.7 Sensitivity and specificity

Cross-classified diagnostic results of the serology test and parasitological urine examination to estimate the sensitivity and specificity are shown in Table 3.3. Only 16 (3.7%) children (10 females and 6 males, all aged ≥5 years) were found egg-positive but classified as infection negative using the serological diagnostic method. In addition, the parasite eggs were detected in only one urine sample for 9 out of these 16 children, and the maximum recorded egg count was 4 eggs/10 mL urine.

Table 3.3: Detection of *S. haematobium* infection status by parasitological examination of eggs in urine and serological testing for IgM antibody response against egg antigens (SEA). Diagnostic test results by age group.

		Serology test		
Age group	**Parasitology test**	+	−	**Total**
1–5 years	+	17	1	18
	−	28	51	79
6–10 years	+	131	15	146
	−	137	58	195
	Total	313	125	438

The results from Bayesian analysis showed that the serological diagnostic technique in comparison to the parasitology test had a higher sensitivity but a lower specificity (estimated with high variability) as illustrated in Table 3.4. The analysis further revealed that the sensitivity of the serological diagnostic technique did not vary by age group or *S. haematobium* infection intensity burden (Table 3.5).

Table 3.4: Comparing the accuracy of serology *vs.* parasitology diagnostic tests for detecting *S. haematobium* infection in the absence of a gold standard. Estimates of posterior mean and median sensitivity and specificity from the Bayesian model with 95% probability intervals (95% PI).

Diagnostic test	Parameter	Mean	Median	95% PI	
Parasitology	Sensitivity	0.648	0.609	0.476	0.948
(Urine examination)	Specificity	0.940	0.942	0.894	0.976
Serology	Sensitivity	0.940	0.941	0.887	0.984
(SEA-IgM)	Specificity	0.645	0.634	0.418	0.925

Table 3.5: Performance of the serology test in detecting infection. Comparing sensitivity of the diagnostic test by age group and infection intensity burden. Estimates of posterior mean and median sensitivity and specificity from the Bayesian model with 95% probability intervals (95% PI).

		Sensitivity of serology test			
Variable	**Subgroup**	**Mean**	**Median**	**95% PI**	
Age group	1–5 years	0.906	0.919	0.758	0.987
	6–10 years	0.915	0.922	0.805	0.988
Infection	Light intensity	0.945	0.947	0.885	0.990
	Heavy intensity	0.927	0.934	0.828	0.989

Infection intensity burden defined according to the WHO classification (WHO 2002).
Light intensity=1–49 eggs/10 mL urine; Heavy intensity ≥ 50*/10 mL urine.*

3.8 Dipstick microhaematuria test

S. haematobium infection prevalence based on dipstick-detected microhaematuria was compared to the prevalence determined by the parasitology test on a subgroup of 190 children (1–5 years, n=51; 6–10 years, n=139). As highlighted earlier, this subset consisted only of those children who microhaematuria dipstick tests measured in addition to the parasitological and serological examinations. The overall infection prevalence based on the presence of microhaematuria

was 86.3% (95% CI: 81.9–91.7%) compared to 37.9% (95% CI: 30.9–44.9%) determined by parasitology test in this subgroup. Furthermore, the results revealed that infection levels based on dipstick-detected microhaematuria were significantly higher compared to those based on parasitology test in both age groups (Figure 3.5). Microhaematuria was detected in all egg-positive children aged 1–5 years old. Four (2.9%) children aged 6–10 years old were found egg-positive but tested negative for microhaematuria detected by the dipsticks. Since a high proportion (Figure 3.5) of children in this subgroup analysis tested positive for dipstick-detected microhaematuria, the age-dependent prevalence could not be precisely estimated using the generalized linear regression model.

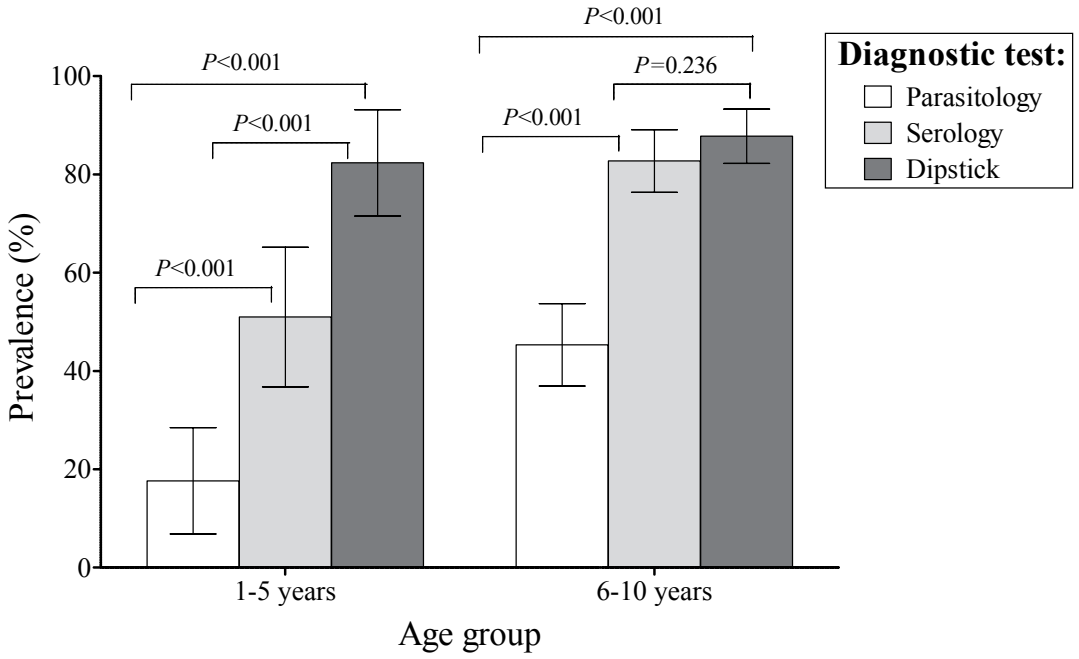


Figure 3.5: Prevalence of *S. haematobium* infection determined using parasitology, serology and dipstick microhaematuria diagnostic test methods. Comparisons by age group on a subset of 190 children (1–5 years, n=51; 6–10 years, n=139). The error bars indicate 95% confidence intervals. The P-values are from χ^2-tests for the differences in prevalence between the diagnostic methods.

Further analysis to evaluate the accuracy of the dipstick microhaematuria test in detecting infection compared with the parasitological technique revealed that the dipstick test had a higher sensitivity (Table 3.6). However, the mean specificity of the parasitology test was higher compared to that of the dipstick

microhaematuria test. The dipsticks performed similarly among children carrying light (n=58) or heavy (n=14) infection intensity burdens both with an overall estimated sensitivity of 94.0%.

Table 3.6: Diagnostic performance of the dipstick test for microhaematuria compared to the parasitology test. Estimates of sensitivity and specificity from the Bayesian model in the absence of a gold standard. Estimates of posterior mean and median sensitivity and specificity from the Bayesian model with 95% probability intervals (95% PI).

Diagnostic test	**Parameter**	**Mean**	**Median**	**95% PI**	
Parasitology	Sensitivity	0.436	0.435	0.364	0.510
(Urine examination)	Specificity	0.945	0.947	0.896	0.979
Microhaematuria	Sensitivity	0.829	0.830	0.799	0.858
(Urine dipstick)	Specificity	0.881	0.884	0.804	0.940

3.9 Discussion

Following successful advocacy by the World Health Assembly in 2001 (WHO 2001*a*), repeated mass drug administration with PZQ has become the key control strategy to combat schistosome infection and prevent development of severe morbidity and is currently implemented in most control programmes in Africa (Fenwick *et al.* 2009; Stothard *et al.* 2013). Since the required treatment regimens for MDA depend on pre-determined infection prevalence levels of the target groups (WHO 2002), it is of clinical and public health importance that sensitive diagnostic tools are used to determine infection. This will facilitate timely treatment of individuals and adequate interventions for targeted populations (Mahfouz *et al.* 2011). Thus in this current chapter, I hypothesized that the levels of *S. haematobium* prevalence determined by serological detection of soluble egg antigens (SEA) would differ to those detected by the parasitological egg examination in urine. In addition, these infection levels were compared between preschool and primary school-aged children to elucidate the need for inclusion of the neglected preschool age group into control programmes. The implications of the prevalence of infection determined using the different diagnostic methods for the WHO recommended treatment regimens were also investigated.

The results of this chapter showed high levels of exposure to schistosomes among preschool-aged children indicated by positive IgM antibody responses against parasite antigens (CAP/SEA) and/or eggs in urine. This finding is consistent with that of Woolhouse *et al.* (2000) who reported evidence of exposure in children as young as four months old. Infection prevalence determined by parasitological and serological diagnostic techniques increased significantly with age. More importantly, the results of this chapter revealed significant infection prevalence among preschool-aged children, further concurring with findings from recent studies also reporting significant schistosome infection burden in this age group (Garba *et al.* 2010; Mutapi *et al.* 2011; Sousa-Figueiredo *et al.* 2010; Stothard *et al.* 2011). These findings corroborate the evidence that in endemic populations young children are at risk of acquiring schistosome infection and may develop severe morbidity if left untreated (Ekpo *et al.* 2012*a*; Stothard *et al.* 2013). The inclusion of these preschool children in the control programmes, as

recently recommended by the WHO (WHO 2011*a*) will facilitate implementation of improved effective and timely interventions (Garba *et al.* 2010).

In contrast to the serology test, the parasitological technique underestimated infection prevalence in both age groups. These findings were also confirmed by the Bayesian estimates for the sensitivity and specificity of the two tests applied to evaluate the diagnostic performance when each test was used independently in the absence of a gold standard test. The reduced sensitivity of the parasitological technique despite repeated urine examinations can be attributed to the fact that the majority of children in this study carried light infection intensities. It was reassuring that the performance of the serological diagnostic test did not vary with infection intensity burden or age group, indicative of its utility as an additional diagnostic tool applicable in untreated preschool-aged children.

Based on the WHO guidelines (WHO, 2002), infection prevalence derived from the serology test suggested a more frequent treatment intervention for this study population compared to that implicated by the parasitological diagnostic technique. These findings reveal the importance of the diagnostic tools used in the field, as these have a bearing on the choice of treatment strategies required for the targeted populations. These WHO recommended treatment strategies for schistosome control are based upon parasitological examinations results, and were developed before the contribution of light infections (often missed by the parasitology test) to the development of morbidity was fully realised. The use of additional diagnostic tools that can detect light infections, as demonstrated in this study, and a better definition of morbidity arising from low schistosome infections (as recently summarized by King & Bertsch (2013)) support the current efforts (Mutapi *et al.* 2011; Stothard *et al.* 2013) and recommendations (WHO 2011*a*) for inclusion of preschool-aged children in schistosome control programmes.

Similar patterns of age-dependent infection prevalence profiles were observed for the parasitological and serological diagnostic techniques, indicative of an early exposure to infection and the accumulation of worm burden as children grow older (Garba *et al.* 2010; Stothard *et al.* 2011). The discrepancy between infection levels determined by the two diagnostic methods also increased with age. Consequently, the observed age-prevalence patterns suggested that the required intervention strategies may vary with age in young children. These above findings are important for the planning of interventions and allocation of resources.

The proportion of schistosome egg-positive children classified as uninfected by

the serological diagnostic technique can theoretically be attributed to two reasons: 1) contamination of the urine samples (Mutapi 2011), this can occur as a result of instruments not being thoroughly cleaned or urine contamination with stool, especially for young female children; and 2) individual variability in mounting an immune response against the parasite antigens (Stothard *et al.* 2011). The high level of technical expertise in conducting the parasitological examinations and repeated urine examinations ensured that technical errors were kept minimal in this study.

The use of dipstick-detected microhaematuria as an indicator of *S. haematobium* infection was restricted to a subset of the study population and the results indicated significantly higher prevalence levels in both age groups compared to infection levels determined by the parasitological diagnostic technique. These results are consistent with findings by King & Bertsch (2013), and highlighted the potential utility of dipstick microhaematuria test as an additional rapid diagnostic tool applicable in preschool children. However, these results need to be interpreted with caution as bias can arise due to other conditions causing microhaematuria in children (e.g. glomerular causes not attributable to schistosome infections (Meyers 2004)).

3.10 Conclusion

The current study showed significant *S. haematobium* infection levels in preschool and primary school-aged children. Infection intensity and prevalence increased rapidly from early childhood, stressing the need for treatment of preschool-aged children. Infection prevalence based on the serological method suggested a more frequent MDA regiment for the population than that suggested by the parasitological diagnostic technique. In conclusion, the serological technique is a valuable diagnostic tool that could be used in conjunction with the parasitological technique to improve the detection of schistosome infections in preschool-aged children. The choice of diagnostic tools used in the field has important implications for treatment strategies required for the control of schistosome infections in young children.

Chapter 4

Identifying and evaluating field indicators of schistosome-related morbidity in children

Part of this work has been published (Wami *et al.* 2015), and a copy of the manuscript is included in Appendix H.

4.1 Introduction

Chronic infection with *S. haematobium*, if left untreated can result in severe clinical morbidity including anaemia, malnutrition, organ-specific complications such as urinary tract and bladder pathology, enlarged liver and spleen, kidney damage, and squamous bladder cancer (Colley *et al.* 2014; Gryseels *et al.* 2006; King 2002; Smith & Christie 1986). Schistosome control programmes focus on preventive chemotherapy with praziquantel to reduce or prevent the development of severe morbidity due to schistosomiasis, and thereby improving the health of the infected individuals and targeted communities (WHO 2010). In order to appropriately plan and implement such interventions, as well as monitor their effectiveness, a better understanding of infection levels and associated morbidity due to schistosomiasis in the target populations is essential. This requires the use of reliable rapid diagnostic tools that can be used in the field (Chen & Mott 1989; van der Werf & de Vlas 2004).

In the past decade, progress has been made towards improving methods of determining schistosome-related morbidity and various techniques have been

evaluated under field conditions (van der Werf & de Vlas 2004). For example, ultrasonography has been shown to be effective in detecting organ-specific morbidity (King *et al.* 2003; Richter *et al.* 2000). Urinalysis has been widely used as a rapid indirect assessment tool for early urinary tract morbidity due to urogenital schistosomiasis (Stothard *et al.* 2009). In addition, standardized questionnaires recommended by the WHO for rapid screening of *S. haematobium* infection and morbidity have been extensively used in endemic regions (WHO 2002). However, most of these studies have focused in older children, typically primary school-aged children (6–10 years), who are the current main targets of schistosome control programmes or adults.

In a major effort to promote child health and development, and improve cognitive potential of children in endemic regions, the WHO recently recommended praziquantel treatment for schistosomiasis in preschool-aged children (aged 5 years and below) through regular health services and their inclusion in ongoing schistosome control programmes (WHO 2011*a*). However, so far the performance of available morbidity diagnostic tools has not been thoroughly evaluated in this age group. In addition, although a few recent studies have been published on the risk of morbidity due to *S. mansoni* infection in young children (Betson *et al.* 2010; Dawson *et al.* 2013), to date there is still paucity of studies quantifying the burden of *S. haematobium*-related morbidity in preschool-aged children.

4.2 Aims

The aims of this chapter are to evaluate the utility of currently available diagnostic tools for detecting schistosome-related morbidity and to determine markers which can be used in the field to identify and quantify morbidity associated with schistosomiasis. To address the knowledge gap about the performance of available morbidity diagnostic tools in endemically exposed children, I have compared the utility of several point-of-care (POC) tools for diagnosing *S. haematobium*-related morbidity, namely: questionnaire-reported haematuria and dysuria, clinical examination, dipstick urinalysis, and measurement of urine albumin-to-creatinine ratio (UACR) in preschool (1–5 years) and primary school-aged (6–10 years) children. I chose the above diagnostic techniques because they are non-invasive and have close biological characteristics. Hence, these tools are suitable for young children and their performances to detect morbidity are

comparable. For example, reported painful urination and haematuria which can be detected using the above tools, is associated with urinary tract morbidity due to *S. haematobium* infection. In addition, presence of chemical components in urine (e.g. albumin) detectable by urinalysis techniques can also be indicative of *S. haematobium* induced kidney and urinary tract morbidity (Webster *et al.* 2009). Ultrasonography is one other non-invasive tool that could be used to detect schistosome-associated morbidity at the point-of-care. However, for logistical reasons (e.g. no power supply at schools) and associated costs ultrasonography was not performed in the current study sample of preschool and primary school-aged children.

This is the first study to validate several indicators of schistosome-related morbidity in untreated preschool-aged children in a single population and addresses the paucity of data on the morbidity burden due to schistosomiasis in this age group.

4.3 Hypothesis

There is no difference in the performance of the currently available techniques for detecting morbidity due to *S. haematobium* infection between children aged 1–5 years and 6–10 year olds.

4.4 Materials and Methods

4.4.1 Study design

Children meeting the study criteria described in chapter 2 (section 2.3.4) were recruited into the study on voluntary basis and were free to withdraw at any time with no further obligation. Figure 4.1 shows the study design flow chart indicating the number of children enrolled in the study and included in the final analysis. For this study, 384 participants were screened as some children in the study population were absent for enrolment during the particular survey days. After sample collection, participants were offered treatment with praziquantel at the standard dosage of 40 mg/kg body weight, administered by the local physician.

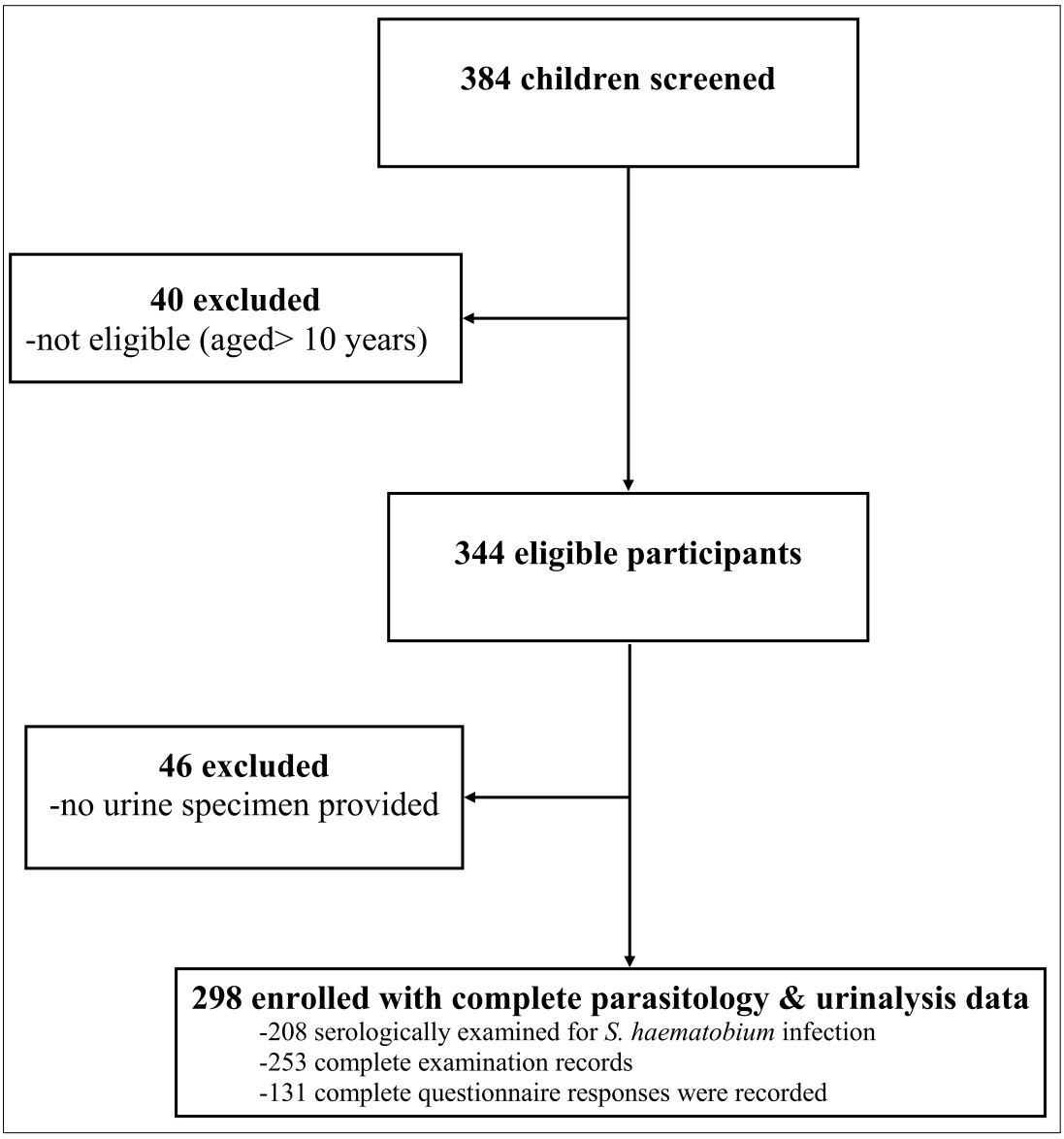


Figure 4.1: Flowchart indicating number of children enrolled in the study and excluded from the final analysis.

4.4.2 Study population

A total of 298 children fulfilled the study criteria (Figure 4.1), and these comprised of 142 (47.7%) males and 156 (52.3%) females, as reported in Table 4.1.

Table 4.1: Demographic characteristics of the study population.

Age group	Sample size (n)	Median age	Sex (Male/Female)
1–5 years	104	4.0 years	58/46
6–10 years	194	8.0 years	84/110
Overall	298	7.0 years	142/156

4.4.3 Determination of schistosome infection status

Urine and stool samples were collected for all the study participants and examined using parasitological methods as already described in section 2.3.5 (chapter 2). A small proportion, 6.0% (n=18) of the children in the current study were found to be positive for *S. mansoni*. I compared the morbidity characteristics of these children to those of a random sample drawn from age and sex matched *S. mansoni* negative children and no differences were observed, hence these children were kept in the study for the final analyses.

In chapter 3, I demonstrated that the parasitology technique lacked sensitivity in detecting light infections in children. Thus, in addition to parasitology, IgM antibody responses directed against soluble egg antigens (SEA) were used in the current chapter to improve the diagnosis of *S. haematobium* infection. Details of the protocols used to quantify the serum antibody levels are described chapter 2. Children were categorized as infected based on serology if their anti-egg IgM antibody levels were more than two standard deviations (SD) above the mean estimated from sera of negative controls, as described in chapter 3 and in the recently published study (Wami *et al.* 2014).

4.4.4 Morbidity measurement

In addition to parasitology, urine samples collected for each of the participants were examined for morbidity due to *S. haematobium* infection detected using the different techniques following the procedures described in chapter 2. In this study, dipstick reagent strips were used to detect the presence of the urine attributes (nitrites, leukocytes, microhaematuria, proteinuria) and measure the physical urine characteristics (pH and specific gravity). Visible haematuria was assessed by inspection of first day urine samples and was characterised as positive

for clearly red urine under the naked eye and negative if the urine sample did not contain visible blood. Questionnaire responses to history/current presence of haematuria and dysuria (yes/no) were used to ascertain the prevalence of self-reported schistosome-related morbidity among children. Parents/guardians responded on behalf of the children aged 5 years and below (section 2.3.9). Following the manufacturer's guidelines (CLINITEK Microalbumin Reagent Strips, Bayer, UK), a high abnormal threshold of urine albumin-creatinine-ratio (UACR >33.9 mg/mmol) was used to ascertain presence of albuminuria, a biological marker of urinary tract infection and an early predictor of progressive kidney disease (Eknoyan *et al.* 2003; Stothard *et al.* 2009). Additionally, results of clinical examination (performed by experienced health personnel) were used to detect the presence of schistosome-related morbidity based on the observed signs and symptoms.

4.5 Statistical Analysis

4.6 Identification of markers of morbidity

The method of non-metric multidimensional scaling (NMDS) was used to identify urinary dipstick markers that contributed most to the differences in schistosome-related morbidity observed in this population following the procedure already described in section 2.7.5 (chapter 2). To test for the univariate associations between different markers of morbidity by sex, age group or *S. haematobium* infection status, I used the method of Chi-square (χ^2) tests. In addition, the phi-coefficient (ϕ) was used to determine the strength of correlation between dichotomous markers (presence/absence) of schistosome-related morbidity.

4.6.1 Relating morbidity to *S. haematobium* infection

Using multiple logistic regressions, I investigated factors influencing the prevalence of schistosome-related morbidity. Each of the morbidity indicators was included as a response variable Y (1=presence or 0=absence), with sex (male *vs.* female), age group (1–5 years *vs.* 6–10 years) and *S. haematobium* infection status (determined by parasitology or serology) or infection intensity, transformed using $\log_{10}$(egg count + 1) included as risk factors in the models. Two-way interaction effects

were included in model building, however, none were found to be significant and hence were subsequently dropped from the final models using the backward method of selection (Agresti 2002). The Akaike information criterion (AIC) was used for the model selection. The Hosmer-Lemeshow test was used for goodness of fit tests of the logistic regression models (Agresti 2002). The model results were expressed as odds ratios (OR), together with the corresponding Wald-test P-values for significance of the parameters. For each binary morbidity response marker Y, the main effects logistic regression was formulated as follows:

$$logit(\pi_{ij}) = \ln\left(\frac{\pi_{ij}}{1-\pi_{ij}}\right) = \beta_0 + \beta_1 \text{Sex} + \beta_2 \text{Age group} + \beta_3 \text{Infection status} \quad (4.1)$$

where,

π_{ij}: is the probability of presence of schistosome-related morbidity at the j^{th} survey period for the i^{th} child.

β_0: is the intercept, and $\beta_1, \ldots, \beta_3$ are the effects of the predictor variables on the binary morbidity response outcome variable.

4.6.2 Morbidity attributable to *S. haematobium* infection

The risk of morbidity in each age group (1–5 years *vs.* 6–10 years) in relation to presence of *S. haematobium* infection was estimated using prevalence ratios, computed by dividing the prevalence of morbidity among infected children by the prevalence in children negative for infection. A prevalence ratio (PR) greater than one indicated a positive association between schistosome infection and presence of associated morbidity. The Breslow-Day test with Tarone's adjustment for small sample sizes (Liu 2005) was used to assess whether the probability of detecting morbidity using the different diagnostic tools in infected children differed between 1–5 years and 6–10 years old children. The population attributable fraction (AFP) and attributable fraction infected (AFI) were used to estimate the proportion of morbidity in the whole study population and among infected children that could be attributed to *S. haematobium* infection, respectively, adjusting for the effects of sex and age group. Furthermore, these estimates were used to compare the utility of the different diagnostic tools for detecting schistosome-related morbidity.

The attributable fraction among the infected was formulated as:

$$AFI = \frac{(PR-1)}{PR} \tag{4.2}$$

The population attributable fraction was calculated as follows:

$$AFP = p \times \frac{(PR-1)}{PR} \tag{4.3}$$

where p is the prevalence of *S. haematobium* infection the children presenting with schistosome-related morbidity.

Approximate 95% confidence intervals were calculated using the method described by Rothman *et al.* (2008). For meaningful interpretations, attributable fractions were only estimated for the morbidity markers with a prevalence ratio (PR) significantly greater than one.

4.7 Results

4.7.1 Schistosome infection levels

The overall prevalence of *S. haematobium* infection determined by parasitological examination was 35.9% (95% CI: 30.4–41.4%). Infection prevalence amongst children aged 6–10 years was 47.9% (95% CI: 40.8–55.0%), and was significantly higher (χ^2=35.0; P <0.001) compared to the infection prevalence in 1–5 years old children: 13.5% (95% CI: 6.8–20.1%). However, there was no significant difference in the prevalence of infection between male and female children (χ^2=0.5; P=0.466). Infection intensity increased significantly with age (r=0.40; P <0.001), with the highest levels observed between the ages of 8–10 years. Of the 298 children in this study, 28.9% (95% CI: 23.7–34.0%) carried light infection intensities and 7.0% (95% CI: 4.1–10.0%) were heavily infected, as defined according to the WHO categorizations (WHO 2002). The prevalence of *S. haematobium* infection determined by serology was higher than that determined by egg counts in both age groups; 1–5 years: 52.9% (95% CI: 38.8–67.1%), and 6–10 years: 84.1% (95% CI: 78.3–89.9%).

4.7.2 Urinary dipstick morbidity markers

Dipstick-detected microhaematuria and proteinuria contributed most to the observed variability in morbidity among children (taking into account urine's physical characteristics, pH and specific gravity) as indicated by the strong correlations in Table 4.2. The distribution of urinary markers differed significantly between *S. haematobium* egg negative and positive children (T=-50.7; P <0.001) and between the two age groups (T=-19.3; P <0.001). However, there were no differences by sex (T=-1.5; P=0.089). Furthermore, the observed differences were evident from the large NMDS ordination output distances between the respective subgroup centres shown in Figure 4.2. Based on serological diagnosis of infection, significant differences were also observed by infection status (T=-14.0; P <0.001), age group (T=-6.5; P <0.001), but not sex (T=-2.5; P=0.068). In addition, microhaematuria and proteinuria alone explained about two-thirds of the overall variability due to differences between infected and uninfected children (detected by either parasitology or serology).

Table 4.2: Non-metric multidimensional scaling (NMDS) correlations (r) between urinary dipstick attributes and the two ordination axes. The coefficient of determination (R^2) indicates the percentage of the overall variability explained. Strong correlations (absolute $r \geq 0.50$) are highlighted and in in bold.

	NMDS Axis 1: 71.4%		**NMDS Axis 2: 22.3%**	
Dipstick marker	r	R^2 (%)	r	R^2 (%)
Blood	**-0.77**	59.3	**0.51**	26.0
pH	**-0.65**	42.3	**-0.68**	46.2
Specific gravity	0.37	13.7	**0.50**	25.0
Proteins	**-0.79**	62.4	0.01	0.0
Leukocytes	-0.2	4.0	0.08	0.6
Nitrites	-0.13	1.7	0.28	7.8

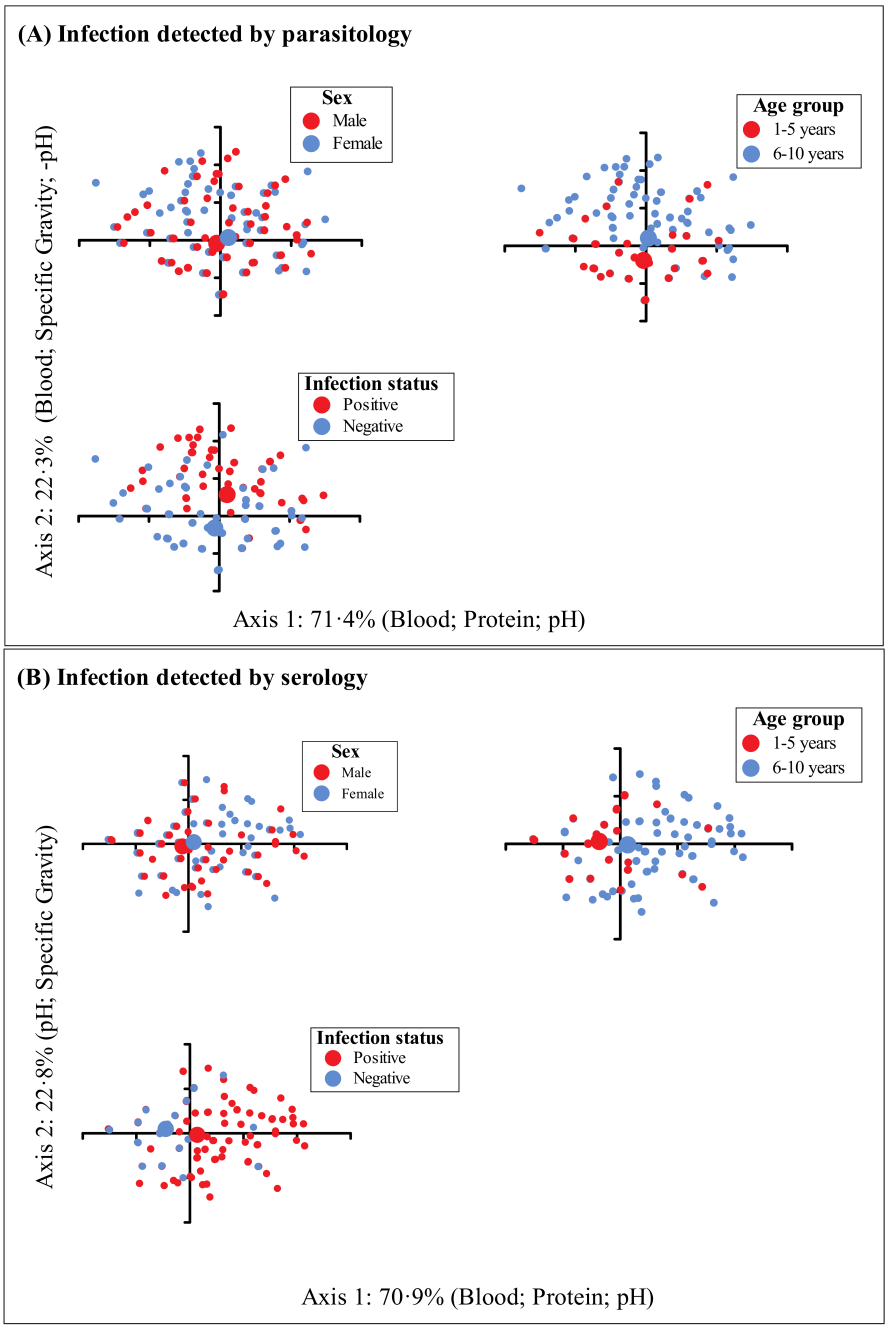


Figure 4.2: Non-metric multidimensional scaling (NMDS) ordination by sex, age-group and *S. haematobium* infection status. Subgroup centres are represented by the bigger closed points, and the distance between these centres is proportional to the level of dissimilarities between subgroups.

4.7.3 Prevalence of morbidity

The prevalence of dipstick-detected microhaematuria was higher than questionnaire-reported haematuria, which in turn was also higher than visible haematuria, as illustrated in Figure 4.3. The morbidity prevalence results illustrated in Figure 4.3 also revealed that children aged 6–10 years tended to report morbidity more frequently compared to parents/guardians of 1–5 years old children. In addition, albuminuria (detected by UACR) and dipstick proteinuria were observed in both age groups (Figure 4.3). A positive association of albuminuria with microhaematuria (ϕ=0.20; P=0.002), or proteinuria (ϕ=0.40; P <0.001) was observed. In comparison to other diagnostic techniques investigated in this study, visual urine inspection, and clinical examination detected the least number of morbidity cases among the children (Figure 4.3).

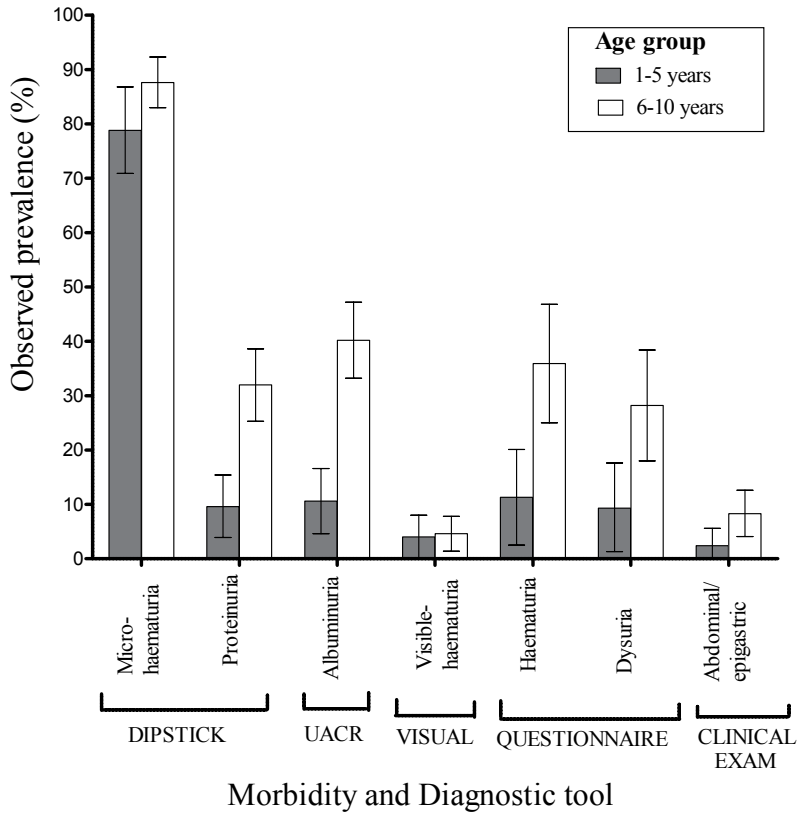


Figure 4.3: Observed prevalences of schistosome-related morbidity by age group, assessed using different diagnostic tools. Error bars indicate the 95% confidence intervals.

4.7.4 Association between schistosome infection and morbidity

Results of multiple logistic regression analyses revealed a significant positive association (in order of strength of the association) of visible haematuria, albuminuria, microhaematuria, and proteinuria with *S. haematobium* infection detected by parasitology as shown in Table 4.3. In addition, *S. haematobium* infection prevalence determined by serology was also found to be significantly associated with albuminuria and proteinuria, adjusting for the effects of sex and age group (Table 4.3). Significant increases in prevalence of albuminuria (OR=5.5; P<0.001), visible haematuria (OR=4.7; P<0.001), microhaematuria (OR=3.4; P=0.005), and proteinuria (OR=3.3; P<0.001) with infection intensity were observed. However, no significant associations between infection intensity and questionnaire-reported haematuria and dysuria, or clinical examination detected morbidity were found.

Table 4.3: Multiple logistic regression odds ratios (OR) to investigate factors associated with the prevalence of morbidity assessed using different diagnostic tools. Investigating the effects of sex (M *vs.* F), Age group (1–5 years *vs.* 6–10 years), and *S. haematobium* infection status (negative *vs.* positive) on morbidity prevalence. Significant effects are highlighted and in in bold.

Morbidity	**Diagnostic**		**Parasitology**[1]		**Serology**[2]	
marker	**tool**	**Variable**	**OR (95% CI)**	***P***	**OR (95% CI)**	***P***
Microhaematuria	Dipstick	Sex	1.8 (0.9–3.4)	0.089	**2.7 (1.1–6.7)**	**0.031**
		Age group	1.2 (0.6–2.4)	0.563	1.4 (0.5–3.9)	0.472
		Infection status	**3.4 (1.5–7.9)**	**0.005**	0.9 (0.3–2.7)	0.902
Proteinuria	Dipstick	Sex	1.2 (0.6–2.1)	0.594	1.2 (0.6–2.3)	0.564
		Age group	**2.5 (1.2–5.5)**	**0.019**	2.0 (0.8–4.9)	0.145
		Infection status	**3.3 (2.2–5.0)**	**<0.001**	**4.5 (1.5–13.6)**	**0.007**
Albuminuria	UACR	Sex	0.8 (0.4–1.5)	0.528	0.8 (0.4–1.5)	0.481
		Age group	**3.1 (1.5–6.7)**	**0.004**	**3.4 (1.3–8.5)**	**0.011**
		Infection status	**5.5 (3.4–8.9)**	**<0.001**	**33.9 (4.5–254.0)**	**0.001**
Haematuria[a]	Visual inspection	Sex	1.1 (0.3–3.6)	0.876	1.4 (0.3–5.9)	0.690
		Age group	0.5 (0.1–1.9)	0.299	1.0 (0.2–5.3)	0.991
		Infection status	**7.8 (1.8–34.4)**	**0.007**	-	-
Haematuria	Questionnaire	Sex	1.0 (0.5–2.4)	0.931	1.6 (0.6–4.2)	0.349
		Age group	**3.9 (1.4-10.8)**	**0.009**	**5.5 (1.1–27.6)**	**0.037**
		Infection status	1.4 (0.6–3.3)	0.443	2.1 (0.4–11.2)	0.385
Dysuria	Questionnaire	Sex	0.6 (0.3–1.6)	0.325	0.6 (0.2–1.4)	0.223
		Age group	**4.1 (1.3–12.6)**	**0.013**	2.5 (0.7–9.1)	0.168
		Infection status	1.0 (0.4–2.4)	0.926	1.6 (0.4–6.9)	0.531
Abdominal/epigastric[b]:	Clinical exam	Sex	0.9 (0.3–2.5)	0.826	1.2 (0.4–3.5)	0.788
		Age group	–	–	–	–
		Infection status	0.9 (0.3–2.6)	0.882	1.2 (0.3–4.4)	0.821

[1]*Infection detected by parasitology.*; [2]*Infection detected by serology.*
[a]*OR not adjusted for serological infection status.*; [b]*OR not adjusted for age group effect.*

4.7.5 Morbidity attributable to *S. haematobium* infection

Since the morbidity markers are not specific to schistosomiasis but are general markers of different physiological and biochemical processes, I went further to determine how much of the morbidity was attributable to schistosome infection. There was no significant difference in the estimated probability of detecting morbidity between 1–5 years and 6–10 years old children using each of the diagnostic tools (Table 4.4). In addition, it was observed from Table 4.4 that morbidity detected by dipsticks (microhaematuria and proteinuria), UACR (albuminuria), and urine inspection (visible haematuria) had prevalence ratios significantly greater than one. Clinical examination detected morbidity had the lowest prevalence ratio (Table 4.4). Furthermore, the results indicated that albuminuria was the dominant marker of schistosome attributable morbidity at population level, as well as amongst infected children (Figure 4.4). Proteinuria and visible haematuria were also found to be highly attributable to schistosome infection among infected children. Although a high crude prevalence of microhaematuria was observed initially, the analyses revealed that a relatively smaller proportion of microhaematuria was attributed to *S. haematobium* infection (Figure 4.4). The attributable fractions among infected children estimated by age group strata (Figure 4.5) showed a similar trend to the overall estimated attributable fractions noted above.

Table 4.4: Estimates of prevalence ratios (PR) weighted by age group for each of the morbidity markers assessed using different diagnostic tools. Breslow-Day test for homogeneity of the probability of detecting morbidity in infected children. Comparisons between 1–5 years *vs.* 6–10 years old children. Prevalence ratios significantly greater than 1 are highlighted and in in bold.

Morbidity	**Diagnostic**	**Parasitology**[1]			**Serology**[2]		
marker	**tool**	**PR (95% CI)**	χ^2	***P***	**PR (95% CI)**	χ^2	***P***
Microhaematuria	Dipstick	**3.3 (1.4–7.9)**	1.4	0.231	0.9 (0.5–2.6)	0.4	0.509
Proteinuria	Dipstick	**1.5 (1.2–1.8)**	0.2	0.666	**1.3 (1.1–1.5)**	2.5	0.114
Albuminuria	UACR	**2.4 (1.9–3.1)**	0.0	0.927	**1.7 (1.4–1.9)**	0.3	0.571
Haematuria	Visual inspection	**1.1 (1.0–1.2)**	0.0	0.989	**1.5 (1.3–1.7)**	a	a
Haematuria	Questionnaire	1.1 (0.8–1.5)	5.1	0.024	1.1 (0.9–1.5)	1.5	0.225
Dysuria	Questionnaire	1.0 (0.8–1.3)	3.1	0.078	1.1 (0.8–1.6)	5.5	0.017
Abdominal/epigastric	Clinical exam	1.0 (0.3–2.9)	3.0	0.081	1.0 (0.9–1.1)	a	a

[1] *Infection detected by parasitology.*
[2] *Infection detected by serology.*
[a] *Test statistic could not be computed.*

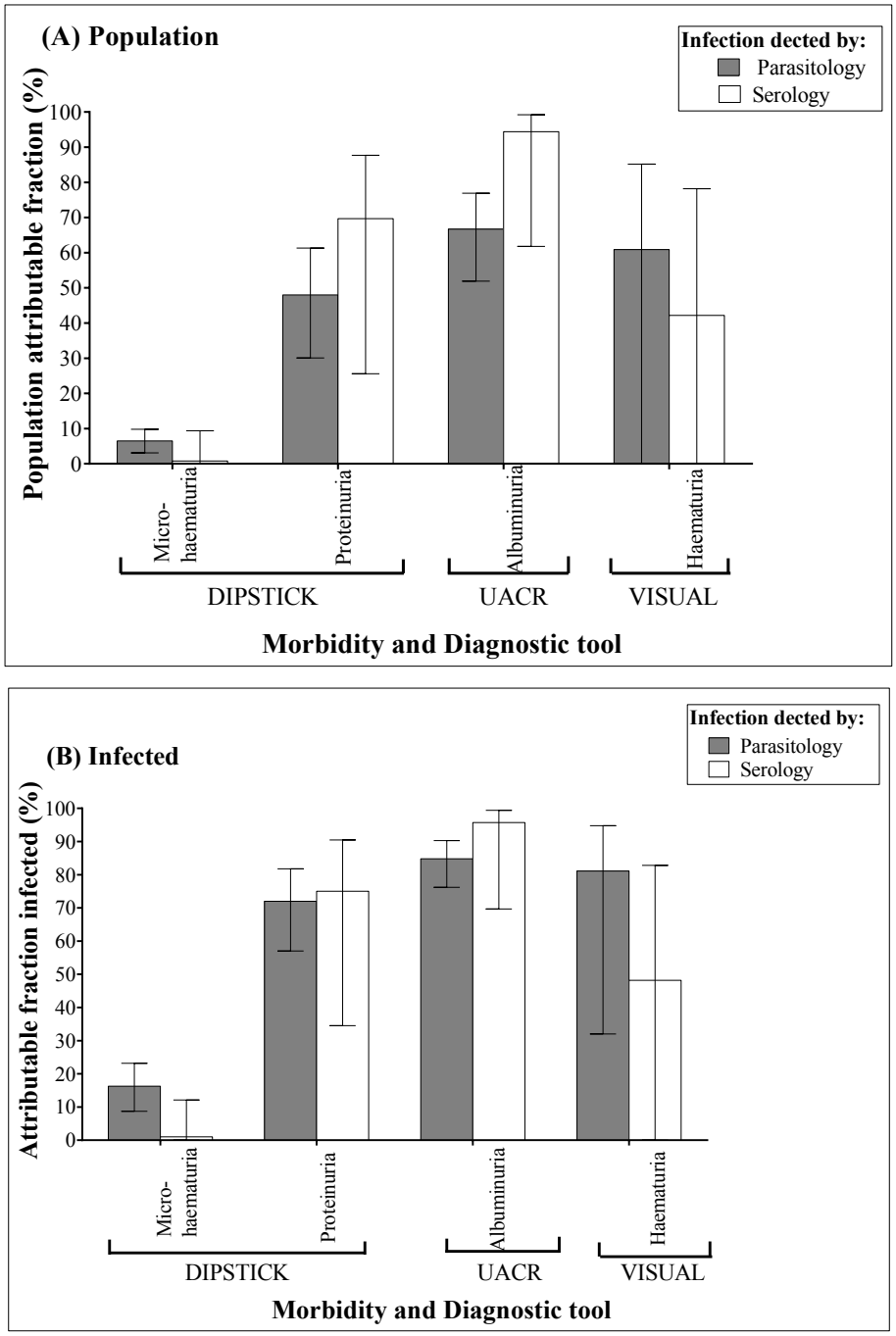


Figure 4.4: Estimated proportion of morbidity attributable to *S. haematobium* infection. (A) Population attributable fraction and (B) Attributable fraction infected.

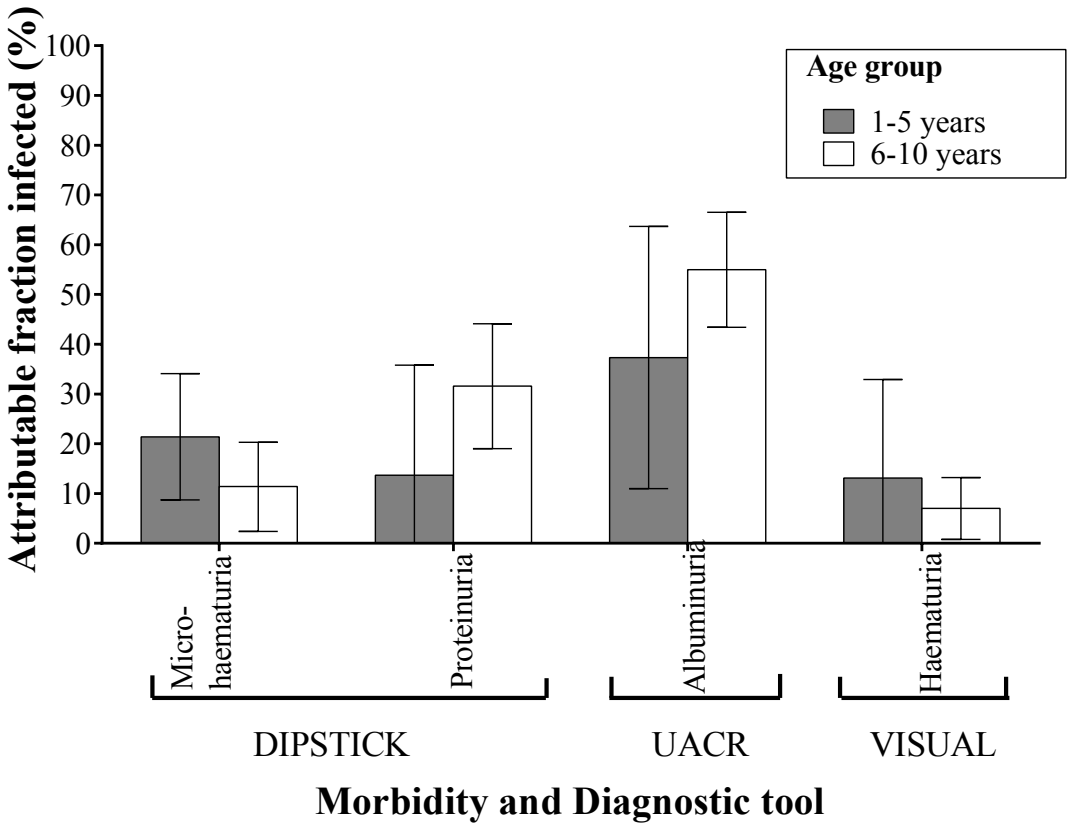


Figure 4.5: Proportion of morbidity attributable to *S. haematobium* infection detected by parasitology, estimated by age group strata.

4.8 Discussion

Until recently, most schistosome control programmes in Africa aimed at reducing development of severe morbidity and improving child health have focused on regular school-based de-worming strategies, targeting children above five years old (Mutapi *et al.* 2011; Sousa-Figueiredo *et al.* 2010; Stothard *et al.* 2011). By focusing treatment on the school-aged population, children of preschool-age have been previously neglected in terms of research and control (Knopp *et al.* 2013). Consequently, less is known about the levels of schistosome-related morbidity in this age-group. Furthermore, research studies evaluating the performance of the current POC markers of schistosome-related morbidity in children aged five years and below are still limited (Betson *et al.* 2010). Estimation of disease burden due to schistosome infections in children has been further complicated by the fact that signs and symptoms commonly associated with schistosomiasis can also be due to other causes (Webster *et al.* 2009). In the absence of a gold standard POC morbidity diagnostic technique, several methods have been used in studies from different endemic settings in older children (aged ≥6 years) and adult populations (van der Werf & de Vlas 2004). In the current chapter, I focused on tools currently used in the field, i.e., the WHO approved questionnaire-based reporting of haematuria and dysuria, clinical examination by qualified clinicians, routinely used dipstick tests measuring several urine attributes, and UACR (for detecting albuminuria) which has previously been evaluated for schistosome morbidity detection (Stothard *et al.* 2009). I evaluated how these tools performed in preschool-aged children (1–5 years) compared to primary school-aged children (6–10 years), who are the current targets of schistosome control programmes.

Results of this current study revealed that children of the two age groups carried quantifiable levels of morbidity as determined by these different diagnostic tools. This finding is in accordance with a recent epidemiological study by Sacko *et al.* (2011) who reported significant prevalence of urinary pathology in endemically exposed children. Of the several urine attributes tested using dipsticks, microhaematuria and proteinuria were significantly associated with *S. haematobium* infection, as has been previously reported in several other studies (Garba *et al.* 2010; Rollinson *et al.* 2005; Stete *et al.* 2012). A high proportion of children aged 5 years and below presented with microhaematuria in this study. More interestingly, the current study demonstrated that the

performance of each of the different POC diagnostic tools for detecting morbidity did not differ between preschool and primary school-aged children infected with *S. haematobium*. These findings are important for planning of future interventions as they provide evidence that preschool-aged children can be effectively screened for praziquantel treatment using the available POC diagnostic tools applicable to older children and adult populations in the field (Lyons *et al.* 2009; Sacko *et al.* 2011).

Since the physical and biological features determined by these diagnostics can arise due to several conditions (Guyatt *et al.* 1995; van der Werf *et al.* 2003), it was determined how much of the proportion of morbidity was attributed to *S. haematobium* infection. Based on the results of prevalence ratios and attributable fractions, UACR was identified as the most reliable tool for detecting schistosome-related morbidity, followed by dipsticks, visual urine inspection, questionnaires and lastly clinical examination. In addition, prevalence of albuminuria determined using UACR was positively associated with presence of microhaematuria and proteinuria detected by dipsticks. This finding suggests that these indicators used in combination can be a better predictor of the presence of urinary tract morbidity due to *S. haematobium* infection in children than using a single test parameter, and thereby facilitating effective and timely interventions. The utility of albuminuria as a valuable indicator of schistosome-related morbidity in our study corroborates earlier findings in school-aged children by Sousa-Figueiredo *et al.* (2009).

Although the proportion of children with visible haematuria was low in this study, it was noted that *S. haematobium* egg-positive children were eight times more likely to present with visible haematuria compared to egg-negative children. In addition, all children with visible haematuria were positive for *S. haematobium* infection detected using the serological diagnostic test. The majority of children in this study carried light infections, and this could explain the observed low prevalence of visible haematuria (King *et al.* 1988).

Since *S. haematobium* infection in endemic areas can easily be inferred from presence of blood in urine, questionnaire responses about recent or current presence of haematuria and dysuria can be used to assess schistosome-related morbidity. However, the current study showed some level of bias in the reporting of haematuria and dysuria between preschool-aged children, where the answers were provided by the parents/guardians and primary school-aged children, who

responded to the questions themselves. One theoretical explanation for these observations could be that children easily mistook concentrated urine as blood in urine, but less likely so by adults and hence resulting in the overestimation of the prevalence of reported morbidity amongst the 6–10 years old children. These results therefore need to be interpreted with caution. Physical clinical markers of morbidity were the least attributable to schistosome infection. These findings are consistent with a recent study by Agnew-Blais *et al.* (2010), who also reported inadequacy of the physical examination method for assessing schistosome-related pathology in school-aged populations.

Nevertheless, there are some limitations when interpreting the results of this current study. Given that approximately 30% of the study participants were not characterized for *S. haematobium* infection based on the more sensitive serological diagnostic technique, caution must be applied when extrapolating the study findings.

4.9 Conclusions

The results of this current chapter confirm that schistosome infection in preschool children does result in significant morbidity. These findings are in agreement with recent studies on *S. mansoni* in Uganda (Betson *et al.* 2010) and *S. haematobium* in Malawi (Poole *et al.* 2014), reiterating the need for antihelminthic treatment in preschool children. This study has gone further to identify morbidity diagnostics with large fractions attributable to schistosome infection, highlighting detection of albuminuria as the best choice for rapid assessment of morbidity attributed to *S. haematobium* infection in children in the field. Finally the study showed that in *S. haematobium* endemic areas, preschool-aged children can be effectively screened for schistosome-related morbidity using the same diagnostic tools applicable to primary school-aged children and adult populations. These findings are of clinical and public health importance, as these tools can be used to identify affected individuals or subgroups, thereby facilitating focused and timely delivery of treatment, as well as evaluate the effectiveness of interventions for improved control.

Chapter 5

Health benefits of biennial *vs.* annual praziquantel treatment in children

5.1 Introduction

In endemic areas children carry the heaviest burden of schistosomiasis due to their frequent contact with infective water (King 2006; Stothard *et al.* 2013; Woolhouse *et al.* 2000). As a consequence, they suffer the most negative impacts of the disease on health such as poor growth, malnutrition, and impaired cognitive development (Colley *et al.* 2014; Gryseels *et al.* 2006). Younger children (≤5 years old) are also passively exposed to infection whilst being washed or bathed with infested water (Stothard & Gabrielli 2007). However, it is only recently that the burden of schistosome-related morbidity has become widely recognized as a significant component to childhood health and development in infants and preschool children (Gurarie *et al.* 2011; Stothard *et al.* 2013; WHO 2011*b*).

In the preceding chapters (chapter 3 and 4), I have demonstrated the presence of significant schistosome infection and related morbidity in children aged 5 years and below, further reaffirming the need for early treatment to prevent development of severe pathology in this age group. Results of my findings and those from recent field studies demonstrating the need for interventions in preschool children (Mutapi *et al.* 2011; Poole *et al.* 2014; Stothard *et al.* 2011; Wami *et al.* 2014, 2015) have heightened the need for a clear knowledge of optimal praziquantel (PZQ) treatment regimens required for this age group

to improve the effectiveness of present interventions (Mutapi 2015). Currently, the WHO recommends different treatment regimens (e.g., annually or biennially) depending on the schistosome infection prevalence in the targeted population, usually assessed by surveying school-aged children (WHO 2002). Thus, having established better diagnostic approach of schistosome infection (chapter 3) and identified markers of schistosome-related morbidity that can be utilized for post-intervention assessment (chapter 4), in the present chapter, I determined the immediate and short-term effects of treatment with PZQ on infection and morbidity by following treated children up to 24 months post-treatment.

5.2 Aims

The main aims of this chapter are two-fold: 1) To investigate the effects of PZQ treatment in children aged 1–5 years old on *S. haematobium* infection and related morbidity levels assessed using the three markers (microhaematuria, proteinuria, and albuminuria) already identified and evaluated in chapter 4; and 2) To compare the impact of biennial *vs.* annual treatments with PZQ on re-infection rates and morbidity attributable to schistosomiasis, assessed over a period of 24 months. In addition, changes in the morbidity indicators in children aged 1–5 years are contrasted with those observed in primary school-aged children (6–10 years) to assess whether repeated PZQ treatment affects the two age groups differently. This is one of the few longitudinal studies investigating the effects of antihelminthic treatment on schistosome-related morbidity in both preschool and primary school-aged children. As such, it provides an initial operational recommendation for future studies on the subject, as well as giving further insights into the health benefits of antihelminthic treatment for preschool-aged children.

5.3 Hypotheses

Based on the analysis of the schistosome infection levels and related morbidity markers: microhaematuria, proteinuria, and albuminuria, this chapter addresses the following null hypotheses:

1. Treatment with praziquantel has no effect on *S. haematobium* infection levels and related markers of morbidity in endemically exposed preschool and primary school-aged children.

2. There is no difference between biennial and annual praziquantel treatment efficacy in reducing re-infection levels and schistosome-related morbidity in children 24 months after treatment.

5.4 Methods

5.4.1 Study area and population

Children meeting the study selection criteria described in chapter 2 were recruited only after the study objectives (including the treatment plan in compliance with study ethics and following the WHO recommendations) had been explained to them and their parents/guardians, and fully informed written consent from parents/guardians had been obtained (see section 2.3.1). Care was taken to identify and exclude children who had been treated as part of the national control programme (mass drug administration) that was active at the same time in the study area. In a recent nationwide survey in school-aged children in Zimbabwe, Midzi *et al.* (2014) reported a *S. haematobium* infection prevalence of 31.2% for Murewa district. This implied that the study area fell in the medium infections level category area as defined by the WHO, and the recommended schistosome treatment regimen is biennial treatment with PZQ of primary school-aged children (see Table 5.1). In high-risk infection areas, the recommended regimen is an annual PZQ treatment of primary school-aged children (Table 5.1). Thus, in this current chapter, I was able to investigate if biennial PZQ treatment was adequate for this current study population of young children.

Table 5.1: The World Health Organization recommended treatment strategies for *S. haematobium* infection targeted at school-aged children. Source: WHO (2002).

Community prevalence	School-based intervention	Health-based and community-based intervention
I. High	Targeted treatment of school-age children, once a year	Access to PZQ for passive case treatment; community directed treatment for high-risk groups recommended
II. Moderate	Targeted treatment of school-age children, once every two years	Access to PZQ for passive case treatment
III. Low	Targeted treatment of school-age children, twice during primary schooling (once on entry, again on leaving)	Access to PZQ for passive case treatment

[a] *PZQ=praziquantel.*

[b] *Prevalence; Low: <10%; Moderate: ≥10% but <50%; High: ≥50%.*

[c] *S. haematobium infections: Light-intensity<50 eggs/10 ml; Heavy-intensity≥50 eggs/10 ml.*

[d] *Passive treatment can be done on presumptive grounds, according to diagnostic algorithms adapted to the endemic situation.*

5.4.2 Study design

As already introduced in chapter 2, the study was designed to relate levels of schistosome infection/re-infection rates and markers of schistosome-related morbidity to the number of praziquantel (PZQ) treatments children received as well as to the different age groups (ages 1–5 years *vs.* 6–10 years) treated in two schools in different villages. The study design is a modification of the traditional treatment re-infection study design where all people are treated at one time point and followed up for specified time period (see Figure 5.2). The modifications were to ensure that the treatment comparative study complied with the appropriate ethical standards protocol.

5.4.3 Ethical statement

Ethical approval to conduct the study was obtained from Medical Research Council in Zimbabwe (MRCZ). Only compliant participants were recruited into the study and were free to drop out at any point during the study. Study enrolment and sample collections were only performed after the study objectives, including the treatment plan in compliance with study ethics and following the WHO recommendations of PZQ treatment regimens had been explained to them and their parents/guardians, and fully informed written consent from parents/guardians had been obtained (see section 2.3.1).

5.4.4 Sampling design and Treatment group allocation

The villages within which the two schools, each with associated early child development centres (ECDs) share the same river systems so that the transmission dynamics in the study area are similar. A preliminary study was conducted on primary school children (grades 3–6 from each school, age range: 6–10 years) to confirm the levels of schistosome infection in the community and study site as per sampling methods for mass drug administration and recommendations by the WHO (Nagelkerke *et al.* 2000; WHO 2006). A clustered sample of 50 compliant children (25 children from each school) were parasitologically screened for schistosome and soil transmitted helminth infections (see Figure 5.1). Children found positive for infection during the pilot survey were treated with a single dose of PZQ (40 mg/kg body weight), but were ineligible for participation in the main study.

In the Zimbabwean primary education system (grade 1–7), for each school grade, children are divided mainly into classes A and B. In addition, children transfer to secondary schools after the seventh grade, therefore, in order to follow children for two years it was essential that the selected primary school children were going to be at the same school for that study period. Thus, only children attending class grades 1–6, in addition to the ECDs (for preschool children) were considered in the present study (see Figure 5.1). The schools also served as feeding centres for children not normally enrolled into any of the educational programmes. Following the initial pre-study survey, pairs of compliant preschool/primary school-aged children were registered and divided by age and sex, and allocated into the two treatment groups (Figure 5.1), recruited at baseline but the initial treatment was administered at different study time points using the following criteria:

1. Selection of annual treatment **Group 1**; recruited at baseline (year 0) and surveyed in year 1 and year 2.
    - For each grade (1–6), select compliant children in A class from each school and assign to treatment Group 1.
    - Select first ECD from each village (associated with the corresponding primary school) and assign to treatment Group 1.
2. Selection of biennial treatment **Group 2**; recruited in year 0 but not screened for parasitology, and also surveyed in year 2.
    - For each grade (1–6), select compliant children attending B class from each school and assign to treatment Group 2.
    - Select second ECD from each village (associated with the corresponding primary school) and assign to treatment Group 2.

The baseline demographic characteristics of the two study groups were similar.

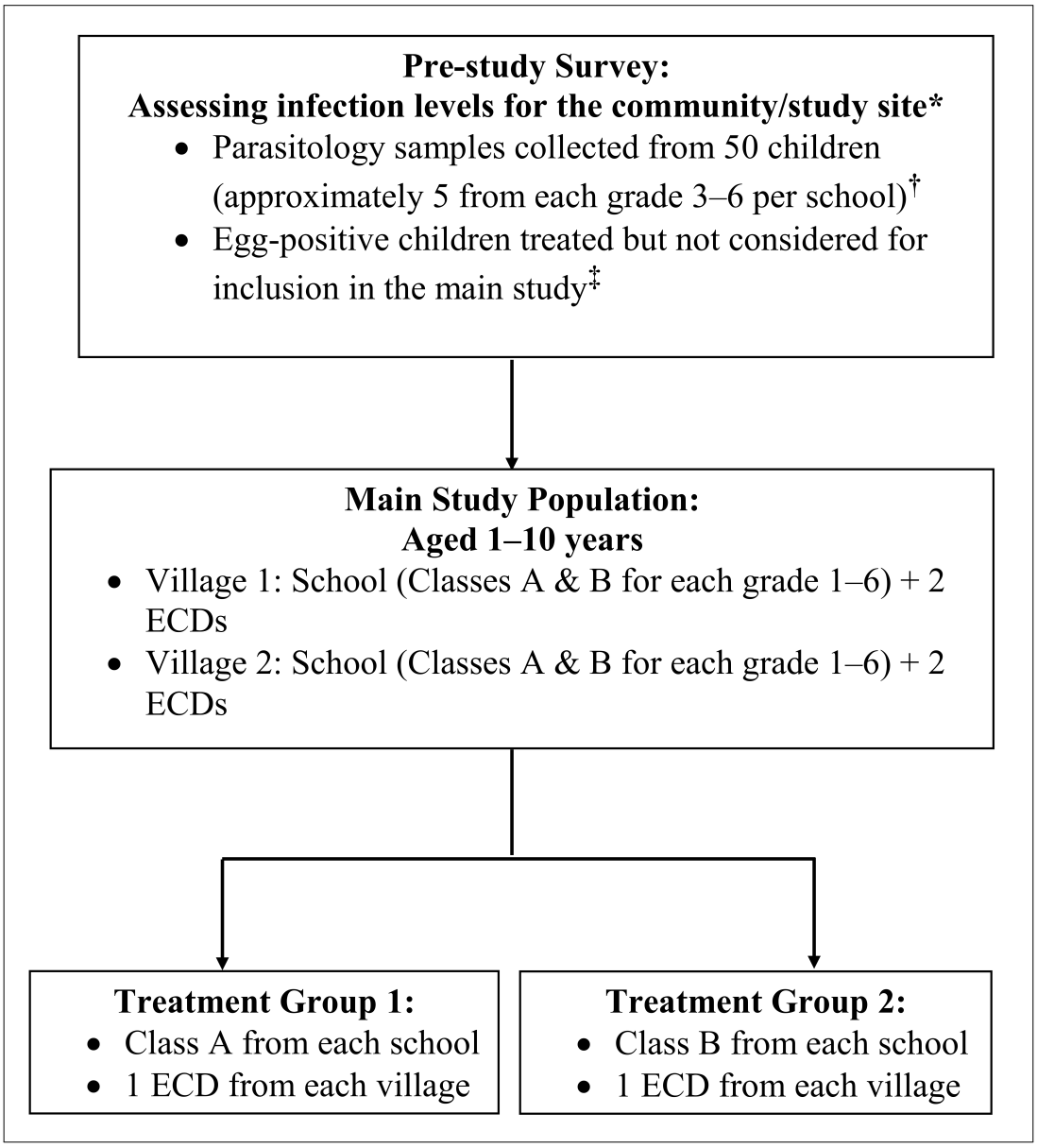


Figure 5.1: Sampling design and allocation of the treatment groups by primary schools and early child development centres (ECDs) within the two study villages.

*Sample sizes for the pre-study survey were based on calculations determined from the recent Zimbabwe National Schistosomiasis Survey (see Midzi *et al.* (2014)).

†Children attending these class grades fall within the age range constituting the high-risk age group for schistosomiasis and STH in the community (WHO 2002).

‡ All children diagnosed positive for infection at any time point were offered antihelminthic treatment with praziquantel.

5.4.5 Study participants

Figure 5.2 shows the sample sizes of the study participants belonging to the different treatment groups included in the final analyses. Treatment efficacy checks were conducted at 12 weeks following initial treatment at baseline and at 12 weeks following treatment administered at the 12 month study time point as shown in Figure 5.2. The timing of the post-treatment efficacy check is important since PZQ is most effective against adult worms (Greenberg 2006). The 12 month period between treatments was chosen to reflect an achievable target for mass chemotherapy in developing countries and in keeping with the recommendations of the 2001 World Health Assembly resolution (54.19) for annual treatment in high infection areas (WHO 2002).

The untreated children consisted of those who would not take Western medication on religious grounds but were willing to take part in the study or were absent on survey treatment days but were voluntarily included in the 12 week follow-up. At the end of the 24 months study, all compliant children were offered antihelminthic treatment with PZQ.

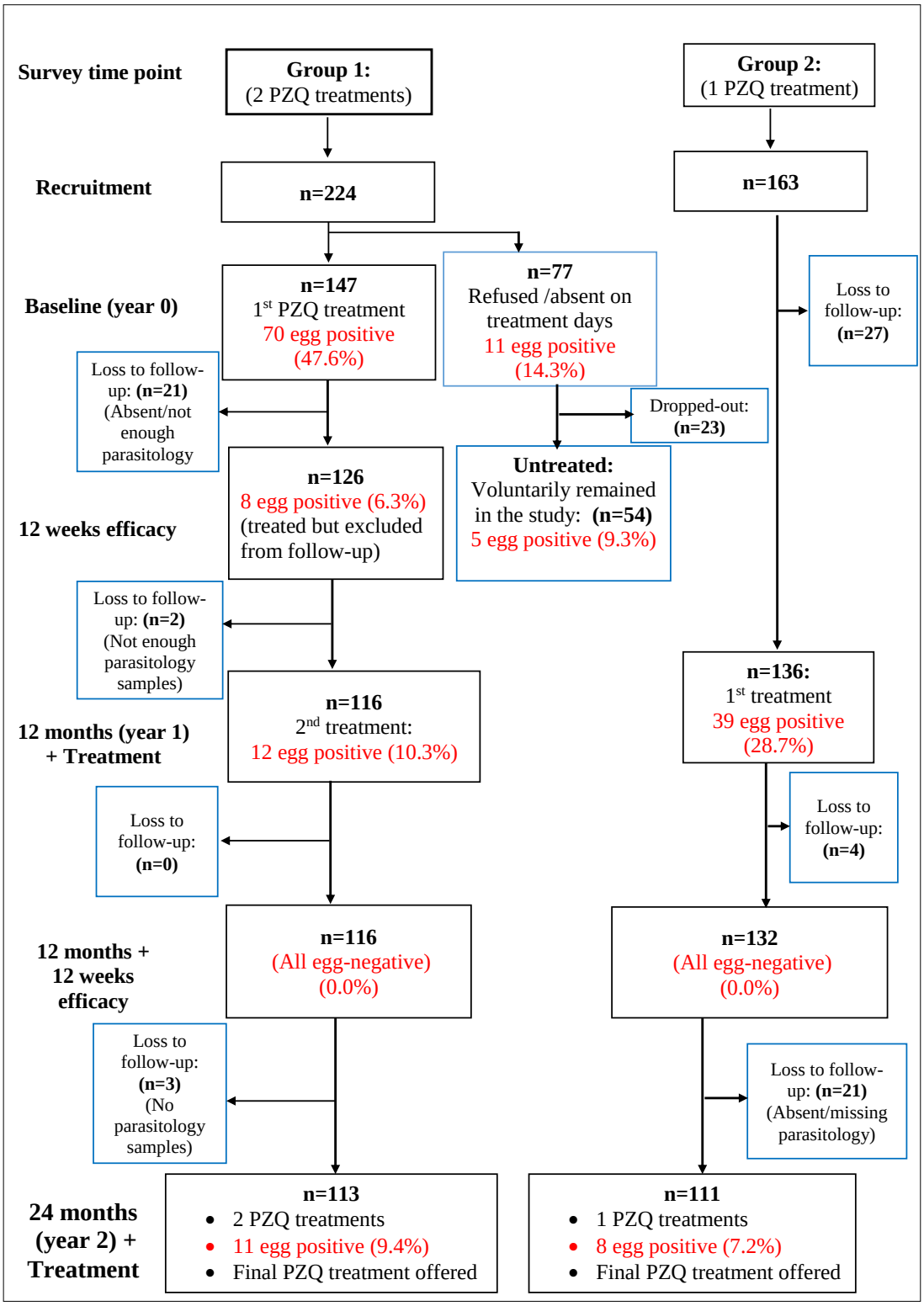


Figure 5.2: Flow chart of the treatment study design. Schematic representation of the treatment effect study showing number of children at baseline and post-treatment follow-up time periods.

5.4.6 Determination of infection status

For each survey period, at least two urine and stool samples were collected for parasitological diagnosis as already described in chapter 2 (section 2.3.5). Since treatment induces changes in IgM antibody response levels, a factor associated with increased exposure of the antigens to the immune system (Mutapi *et al.* 2003; Naus *et al.* 1998), the serological diagnostic technique described in Chapter 3 was not applied in this study.

5.4.7 Identifying markers of schistosome-related morbidity

In chapter 4, I evaluated several different point-of-care morbidity diagnostics and identified microhaematuria, proteinuria, and albuminuria as the main markers showing the largest proportion of morbidity attributable to *S. haematobium* infection. Therefore, these three morbidity markers were selected for the current follow-on study on effects of PZQ treatment.

5.5 Statistical analyses

5.5.1 Data

The descriptions of variables measured in this study are displayed in Table 5.2.

5.5.2 Assessing treatment efficacy on infection levels

I assessed treatment efficacy against *S. haematobium* infection by means of cure rates (CR) and egg reduction rates (ERR), limited to children who were present at both baseline and 12 weeks follow-up, and defined as:

- **CR**=(number of children egg-positive before treatment and confirmed egg-negative at 12 weeks post-treatment follow-up/number of children confirmed egg-positive before treatment and followed up at 12 weeks) X 100;
- **ERR**=(arithmetic mean egg count for the study group before treatment – arithmetic mean egg count for the study group at 12 weeks post-treatment/ arithmetic mean egg count for the study group before treatment) X 100.

Table 5.2: Summary of variables measured in the study.

Variable	Description
StudyID	Unique individual identification number for each participant.
Age	Age of the participant at baseline or age group (1–5 *vs.* 6–10 years).
Sex	Gender of the participant (M *vs.* F).
Infection intensity	Arithmetic mean egg counts/10 mL urine.
Infection status	Infection diagnosed by parasitological egg count (positive/negative).
Survey period	Pre- and post-treatment study periods
Treatment Group	Treatment group (Group 1=annual; Group 2=biennial treatment).
Morbidity	Schistosome-related morbidity outcomes indicated by presence/absence each of the markers microhaematuria, proteinuria or albuminuria.

5.5.3 Assessing the effect of treatment on morbidity levels

The main outcome of interest in this study was whether PZQ treatment has an effect on the levels of schistosome-related morbidity as measured using the three urinalysis markers: microhaematuria, proteinuria, and albuminuria (Table 5.2). The main design variables included host factors sex and age group as described in Table 5.2. To determine whether schistosome-related morbidity prevalence decreased after treatment and to investigate the effect of biennial *vs.* annual PZQ treatments between the two age groups, I used the method of generalized linear mixed models (GLMMs) described in section 2.7.3. Since the allocation of children into different treatment groups was implemented at village level as opposed to individual level, the GLMM technique adjusts for clustering by allowing both fixed and random effects. Briefly, the GLMM was used to model the log odds of the probability of a child presenting with morbidity, as assessed pre- and post-treatment. A random intercept was included in the model to account for possible correlation between children belonging to the same study group. The GLMM to assess the effect of PZQ treatment on prevalence of schistosome-related morbidity at 12 weeks post-treatment was formulated as follows:

$$ln(\frac{\pi_{ij}}{1-\pi_{ij}}) = \beta_0 + \beta_1 \text{Sex} + \beta_2 \text{Agegroup} + \beta_3 \text{Treat} + \beta_4 \text{Agegroup} * \text{Treat} + \text{b}_i \quad (5.1)$$

where π_{ij}: is the probability of presence of morbidity at the j^{th} study time period (pre- or post-treatment) for the i^{th} child, β_0 is the intercept, $\beta_1, \ldots, \beta_4$ are the regression parameters, reflecting the effect of the explanatory variables on the response, b_i: is the random effect term to account for the association repeated observations within each child/treatment group, where $b_i \sim N(0, \sigma_b^2)$ is assumed. F-tests and t-tests from the GLMMs were used to test for overall significant effects and for pairwise comparisons, respectively. In all the analyses, pairwise comparisons were adjusted for family-wise type I error using the less conservative (i.e., has low rate of false negatives) simulation-based approach (Edwards & Berry 1987).

5.6 Results

5.6.1 Treatment efficacy on infection levels at 12 weeks post-treatment

The baseline (Year 0) mean infection intensity in this study population determined by parasitological egg count in urine was 14.0 eggs/10 mL urine (range: 0.0–1013 eggs/10 mL urine). In Group 2, the pre-treatment (Year 1) mean infection intensity was 9.5 eggs/10 mL urine (range: 0.0–195.0 eggs/10 mL urine). PZQ was highly efficacious at reducing *S. haematobium* infection 12 weeks after treatment, with high cure and egg reduction rates observed in both age groups of children belonging to the different treatment groups (Table 5.3). All children belonging to treatment Group 2 had successful curative treatment 12 weeks post-treatment. For the two treatment groups, the observed cure rates at 12 weeks post-treatment did not differ between children aged 1–5 years and the 6–10 year olds (Group1: χ^2=1.33; P=0.248; Group 2: χ^2=0.17; P=0.675). In addition, it was further noted that of the 14 heavily infected children in Group 1 at baseline, 85.7% (n=12) had successfully cleared infection 12 weeks after treatment. Of the remaining 2 children, 1 had a change in mean infection intensity from baseline of 533.5 to 5.0

eggs/10 mL urine, and for the other child, infection intensity decreased from 65.5 eggs/10 mL urine at baseline to 1.0 egg/10 mL at 12 weeks after treatment.

Table 5.3: Praziquantel efficacy in reducing *S. haematobium* infection levels according to age group. Treatment assessed at 12 weeks after initial treatment among children belonging to the different treatment groups.

		Prevalence (%)		Egg count/10 mL		Treatment efficacy	
Treatment	**Age group**	**Before**	**12 weeks**	**Before**	**12 weeks**	**%ERR**	**%CR**
Group 1	1–5 years	29.4	0.0	3.1	0.0	100.0	100.0
	6–10 years	49.5	7.3	26.4	0.2	99.2	91.6
Group 2	1–5 years	17.2	0.0	4.8	0.0	100.0	100.0
	6–10 years	38.1	0.0	12.4	0.0	100.0	100.0

Treatment: Group 1: annual treatment; Group 2: biennial treatment.
Before: baseline/year 1 (for Group 2)
Prevalence=proportion egg positive pre- vs. post-treatment.
CR=cure rate; ERR=egg reduction rate.

5.6.2 Effect of annual *vs.* biennial PZQ treatment on infection levels

Infection levels 12 months after treatment were significantly lower compared to baseline levels for both treatment groups as shown in Figure 5.3. The effect of PZQ treatment on overall infection prevalence levels 12 months after treatment did not differ significantly (OR=0.42; χ^2=0.70; P=0.405) between children receiving annual (Group 1) *vs.* biennial (Group 2) PZQ treatments. None of the re-infected children at 12 months post-treatment carried heavy infection intensities, and the maximum recorded intensity was 27.5 eggs/10 mL urine for a child belonging to treatment Group 1. The odds of re-infection among 1–5 year olds also did not differ significantly: OR=0.76 (95% CI: 0.24–2.47; P=0.652) to those of children aged 6–10 years old, adjusting for sex and treatment group. The overall rate of re-infection 24 months after treatment for children receiving the annual PZQ treatment regimen (Group 1) remained low 9.7% (95% CI: 4.3–15.2%) and was not significantly different (χ^2=2.14; P=0.143) from the re-infection levels observed at 12 months following initial treatment for children in Group 2 receiving biennial treatment. The maximum recorded infection intensity at year 2 study time period was 8.7 eggs/10 mL urine.

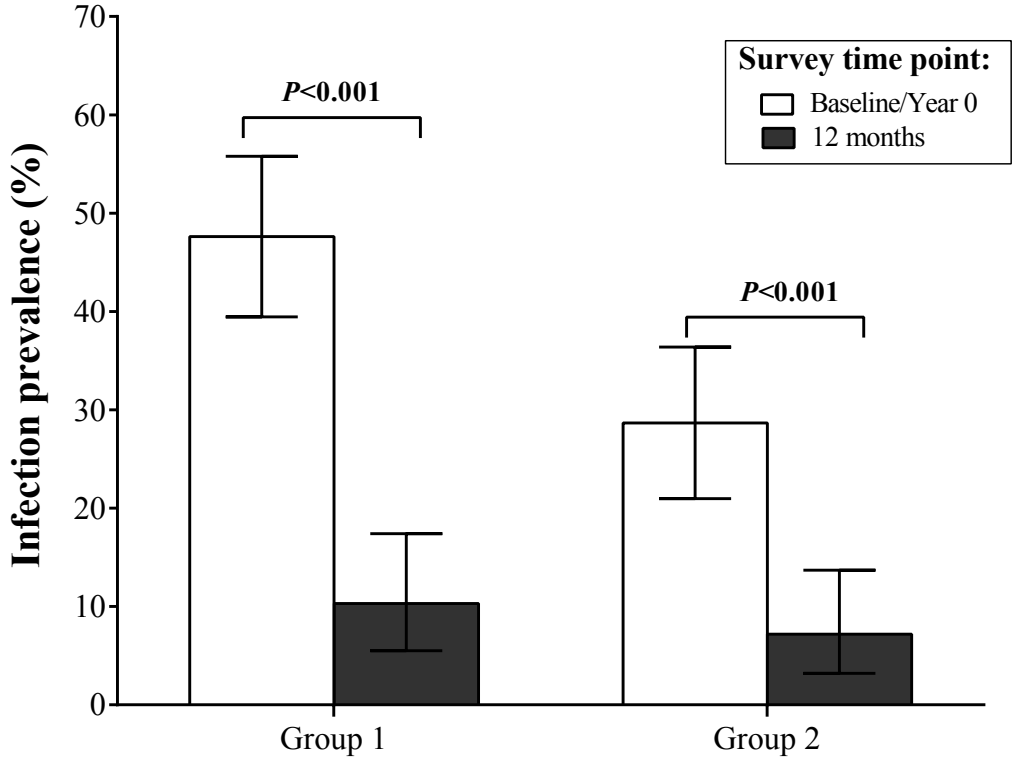


Figure 5.3: Praziquantel efficacy in reducing infection levels in children belonging to different treatment groups. Comparing infection levels at baseline and 12 months after treatment. Group 1: annual and Group 2: biennial PZQ treatment. Error bars indicate 95% CI.

5.6.3 Short-term effects of praziquantel treatment on morbidity levels

At 12 weeks post-treatment, data on markers of schistosome-related morbidity were available for children belonging to Group 1 and untreated controls. Among the treated children at 12 weeks, PZQ treatment significantly reduced levels of proteinuria and albuminuria, but no change was observed for microhaematuria (Figure 5.4). In addition, the odds of a treated child presenting with morbidity 12 weeks post-treatment did not significantly differ by age group (1–5, n=34; 6–10 years, n=92): microhaematuria (odds ratio, OR=0.57; 95% CI: 0.13–2.55), proteinuria (OR=1.61; 95% CI: 0.54–4.76), and albuminuria (OR=0.84; 95% CI: 0.21–3.35). In untreated children who voluntarily remained in the study at 12 weeks, there was no significant change in morbidity prevalence as indicated by the levels of the three markers of schistosome-related morbidity in Figure 5.4.

At 12 months post-treatment, the levels of the three markers of schistosome-related morbidity among treated children receiving annual PZQ treatment (Group 1) were significantly lower compared to baseline levels (Figure 5.5). The effect of treatment on the markers of morbidity did not differ by age group: microhaematuria (OR=1.69; 95% CI: 0.49–5.76; P=0.398), proteinuria (OR=0.69; 95% CI: 0.24–1.99; P=0.494), and albuminuria (OR=0.67; 95% CI: 0.19–2.41; P=0.534).

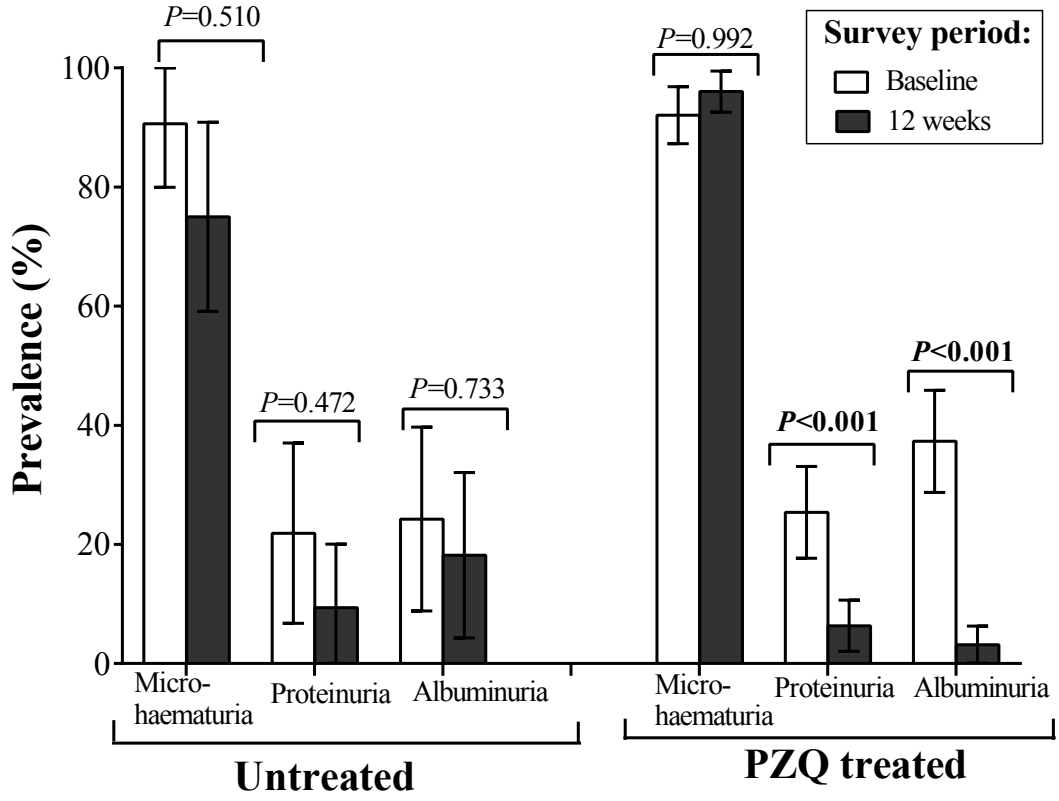


Figure 5.4: Praziquantel (PZQ) effect on prevalence of schistosome-related morbidity. Baseline versus 12 weeks comparisons by treatment group. The error bars indicate the 95% confidence intervals. The *P*-values are from generalized linear mixed models investigating the probability of a child presenting with morbidity pre- and post-treatment, adjusted for host factors sex and age group.

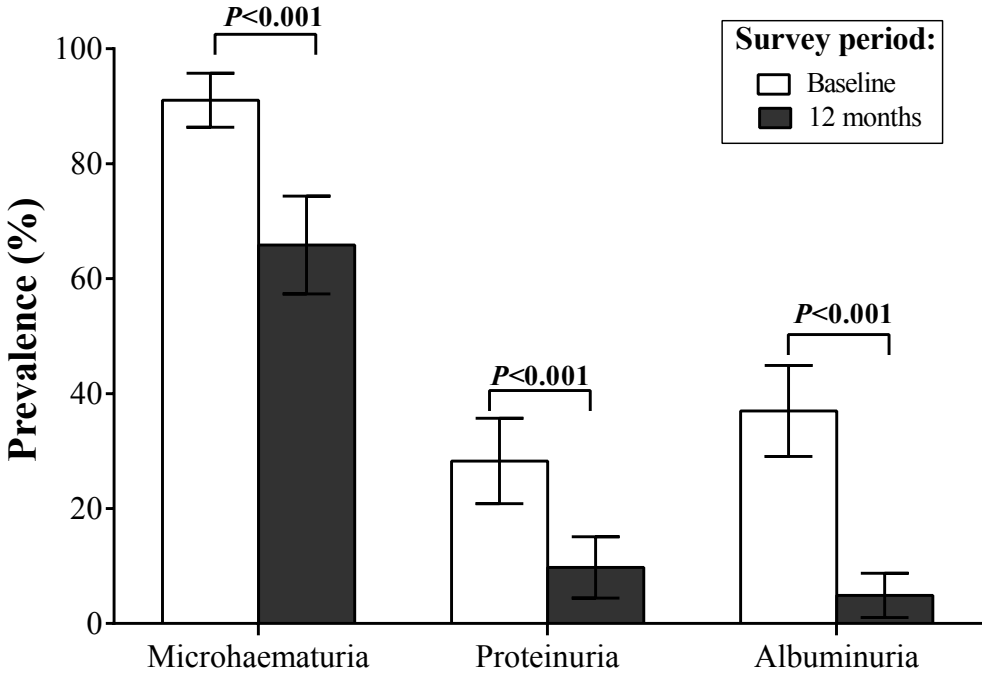


Figure 5.5: Effect of single praziquantel treatment on schistosome-related markers of morbidity at 12 months post-treatment. The error bars indicate the 95% confidence intervals. P-values were obtained from generalized linear mixed models, adjusted for the effects of the host factors sex and age group.

5.6.4 Effect of biennial *vs.* annual PZQ treatment on morbidity

Following the first treatment administered at baseline (start of the field study), children in Group 1 children received an additional PZQ treatment at 12 months, whilst Group 2 children received their first treatment at 12 months survey (see Figure 5.2). At the end of the 24 month study period, I investigated whether within each treatment group (Group 1 *vs.* Group 2), the prevalence of the markers of schistosome-related morbidity significantly changed from pre- *vs.* post-treatment (Figure 5.6). The results of the GLMM analysis showed that treatment significantly reduced levels of microhaematuria and albuminuria in both treatment regimen groups (Figure 5.6). In the case of proteinuria, there was a significant reduction relative to baseline in Group 1 children receiving the annual treatments, but not in the Group 2 children who received the single biennial treatment (Figure 5.6). This was despite the significant initial reduction in proteinuria levels observed in treated children 12 weeks after treatment as shown in Figure 5.4. In addition, at the end of the study period, the odds of presenting with morbidity assessed using the three urinary markers did not differ between children receiving single biennial and annual PZQ treatment regimens (Figure 5.6). When comparing the effect treatment across the two age groups, the results also showed that for children receiving biennial treatment, the odds of presenting with morbidity after the treatment did not differ by age group to those of children who received the annual PZQ treatment regimen (Table 5.4).

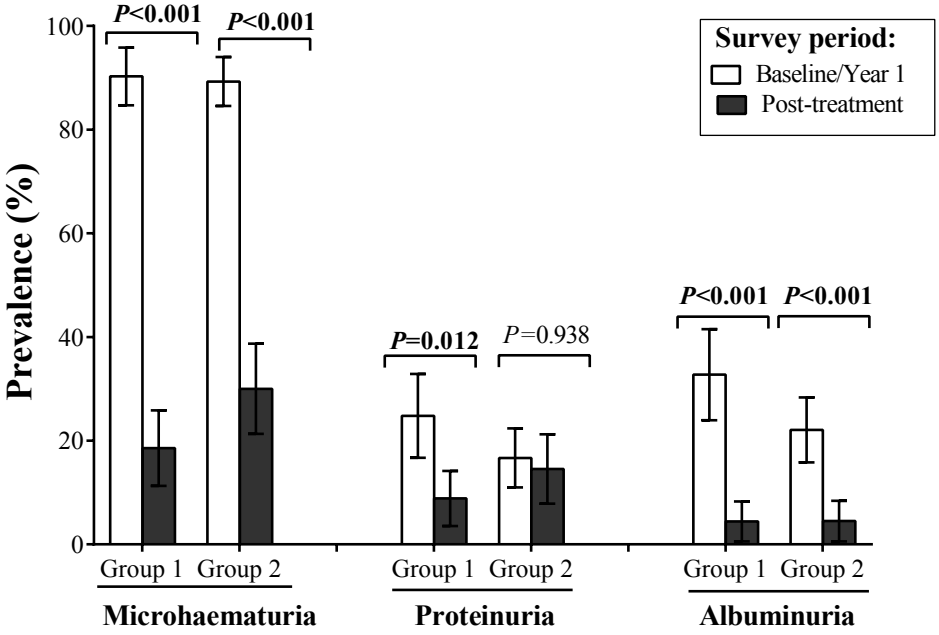


Figure 5.6: Effect of biennial and annual praziquantel (PZQ) treatments on schistosome-related morbidity. Levels of morbidity markers at baseline (year 0) for Group 1 receiving annual treatments or year 1 for Group 2 (biennial treatment) were compared to those at end of the 24 month study period (Year 2). The error bars indicate the 95% confidence intervals. The P-values adjusted for pairwise comparisons were obtained from generalized linear mixed models comparing levels of morbidity markers between treatment groups, allowing for the effects of sex and age group.

Table 5.4: Adjusted odds ratios of schistosome-related morbidity following treatment for children who received a biennial *vs.* annual praziquantel treatment regimens at the end of the study. Comparisons by age group. Group 1 (1–5 years: n=20; 6–10 years: n=93) and Group 2 (1–5 years: n=49; 6–10 years: n=62)

Response marker	Age group	Treatment regimen	OR (95% CI)	*P*-value
Microhaematuria	1–5 years	Annual *vs.* Biennial	0.68 (0.17–2.78)	0.892
	6–10 years	Annual *vs.* Biennial	0.87 (0.39–1.95)	0.969
Proteinuria	1–5 years	Annual *vs.* Biennial	1.07 (0.21–5.51)	0.916
	6–10 years	Annual *vs.* Biennial	0.80 (0.38–1.67)	0.861
Albuminuria	1–5 years	Annual *vs.* Biennial	1.24 (0.16–9.35)	0.992
	6–10 years	Annual *vs.* Biennial	0.83 (0.39–1.74)	0.912

OR=odds ratio.
Response marker=presence/absence of morbidity marker.
Annual treatment (Group 1) ; Biennial treatment (Group 2).

5.7 Discussion

PZQ is currently the antihelminthic drug of choice for treating schistosome infections and reducing related morbidity. Early treatment of schistosome infections in childhood may prevent the development of severe chronic morbidity in adulthood (Richter 2003). In endemic areas, the WHO has recommended frequent PZQ administration to school-aged children delivered at specific time intervals as part of the intervention strategies for existing programmes aimed at controlling subtle morbidity and preventing the development of chronic disease due to schistosomiasis (WHO 2002, 2006). More recently, the recommendation by the WHO has been extended to include preschool children (aged ≤5 years old) in control programmes (WHO 2011*a*). However, it remains to be determined if this age group can just be integrated into the existing treatment regimen or if the optimal treatment regimen for this age group differs from older children. The aims of this chapter were to determine the impact of PZQ treatment and compare the effectiveness of biennial *vs.* annual PZQ treatments in reducing infection and schistosome-related morbidity in endemically exposed children. Short-term general health benefits of PZQ antihelminthic treatment in children of school-age have already been documented (King 2006), therefore by including the primary school-aged children (6–10 year olds) in this study, I was able to determine if the effect of PZQ treatment on schistosome-related morbidity measures was age group-dependent.

As already indicated in previous chapters (chapter 3 and 4), my findings on infection and morbidity in preschool-aged children further support the premise that if left untreated, these children are at an increased risk of developing severe morbidity which may have serious consequences on child development and future quality of life (Dabo *et al.* 2011; Stothard *et al.* 2011). My study results at 12 weeks after chemotherapy for both treatment groups indicated that PZQ was highly efficacious against *S. haematobium* infection. The high cure rates and egg reduction rates observed in this present study are consistent with findings from previous studies that have also reported high treatment efficacy rates within six weeks after treatment (Coulibaly *et al.* 2012; Mutapi *et al.* 2011; Tchuente *et al.* 2004). Interestingly, my study further revealed that PZQ treatment was equally efficacious in reducing *S. haematobium* infection levels in preschool-aged children compared to their older counterparts (6–10 year olds), further

supporting their inclusion in current schistosome control programs (Stothard *et al.* 2013; WHO 2011*a*). Re-infection rates remained significantly low, with marked reduction in infection intensities 12 months and 24 months (for Group 1) after treatment. The results of comparisons of biennial *vs.* annual PZQ treatment revealed beneficiary effects of both treatment regimens on infection levels, with no significant differences observed between children belonging to the two treatment groups. These results suggested that a biennial treatment regimen (following the WHO guidelines for moderate infection prevalence areas) was sufficient in reducing infection levels in this population.

Since schistosome-related morbidity is cumulative and progressive (King 2007), a decrease in current morbidity can reduce the long-term schistosomiasis sequelae. At 12 weeks after the first treatment, there was a significant decrease in the prevalence of morbidity markers among children successfully treated for infection. The current study also showed that the prevalence of morbidity diagnosed by microhaematuria declined slowly, with a significant reduction being observed after 12 months post-treatment. These results showing persistently high levels of microhaematuria differ from some published studies that reported a considerable drop in microhaematuria within 8 weeks after treatment (Nkulila *et al.* 1999; Sacko *et al.* 2009; Stete *et al.* 2012). However, these studies focused on primary school or older children who may have developed chronic infection. Another possible explanation for this delayed decrease in microhaematuria may be that most of the observed microhaematuria in these children may have been due to other conditions not attributable to schistosome infection. In the preceding chapter, I demonstrated that the proportion of microhaematuria attributable to schistosome infection was lower compared to that of albuminuria and proteinuria (Chapter 4). The current study further revealed that a single PZQ treatment dose had sustained effects on the reduction of schistosome-related morbidity, as indicated by the prevalence of albuminuria that remained low after 24 months in children receiving the single treatment. Furthermore, it is interesting to note that despite the fact that children belonging to biennial treatment regiment only received treatment a year later, they showed improved beneficial treatment effects in terms of reduced re-infection rates and prevalence levels of schistosome-related morbidity markers catching up with children who received treatment earlier (annual treatment group).

In view of the current observations based on this study population, it is

practically possible for control programmes in areas of moderate endemicity targeting preschool-aged children to be implemented using the existing treatment strategies designated for school-aged children (WHO 2002). Nevertheless, certain limitations of the study must be considered when interpreting these results. Firstly, since most of the children carried light infections in my study, the parasitological cure rates at 12 weeks post-treatment efficacy check might have been overestimated. However, it is reassuring that the efficacy rates (cure and egg reduction rates) reported in my study are still within the range of those observed from other previous epidemiological studies (Garba *et al.* 2013; Mutapi *et al.* 2011). Secondly, children belonging to the two treatment regimen groups were treated at different time points during the study. Thus, caution must be applied, as my findings might not guarantee extrapolation strength to other study designs. Lastly, participant drop-outs as well as other recruited children preferring not to receive treatment for reasons highlighted in this study or having received treatment through ongoing mass drug administration (MDA) programmes (hence not considered in the current study) could have introduced additional uncertainties in the levels of infection and schistosome-related morbidity markers leading to a potential bias in the effects of treatment reported in this study. Although a random effect was included in the statistical models to account for some of this uncertainty, these results should be interpreted with caution.

5.8 Conclusions

The findings described in this chapter are important for practitioners, policy makers and stakeholders involved in the control of schistosomiasis and timely because of the current global drive to address the health iniquity created by the paucity of information on the impact of PZQ treatment on schistosome-related morbidity in children aged 5 years and below. The results of the current chapter indicate that PZQ treatment not only effectively reduces schistosome infection levels, but also the levels of schistosome-related morbidity in both preschool and primary-school aged children, with marked reduction in morbidity levels being recorded within the first year of treatment and sustained over a period of two years. The study also demonstrated health benefits of biennial praziquantel treatment regimen in reducing schistosome infection and related morbidity were comparable to those observed in children receiving two annual treatments in

this current population. Thus, in conclusion, the treatment regimens currently designated for school-aged children aimed at controlling schistosome infections and morbidity are adequate and applicable to preschool-aged children.

Chapter 6

Evaluating serological biomarkers of inflammation in urogenital schistosomiasis in children

Part of the work in this chapter is in preparation for submission for peer review and publication.

6.1 Introduction

Infection with *S. haematobium* in children commonly manifests itself with blood in urine (haematuria) resulting from physical damage to the blood vessels and the lining of the urogenital tract by parasite eggs exiting the body, as has been introduced in chapter 1. Some of the parasite eggs get trapped in tissues, provoking granulomatous inflammatory host immune responses that can eventually lead to severe urogenital tract pathology, renal complications (hydroureter and hydronephrosis), and squamous cell carcinoma of the bladder (Colley *et al.* 2014; Gryseels *et al.* 2006; Smith & Christie 1986). Recent reports on the global burden of disease indicate that schistosomiasis is responsible for an estimated 3.3 million disability-adjusted life years (Murray *et al.* 2012). In young children, urogenital schistosomiasis can also cause a wide range of health-related conditions (see section 1.5) including lethargy, anaemia and undernourishment which can occur in infected individuals regardless of the level of their infection (King & Dangerfield-Cha 2008; King *et al.* 2005; WHO 2001*b*). Malnutrition and anaemia are associated with impaired child growth, reduced physical fitness, and poor

cognitive and mental development (Friedman *et al.* 2005; Haas & Brownlie 2001; Shaw & Friedman 2011). However, these clinical manifestations of schistosomiasis may be mild and hence go unrecognised in some children (Knopp *et al.* 2013). Continued efforts and new strategies to identify affected children earlier during the course of infection are thus needed in endemic areas.

The main objective of the current control programmes as recommended by the World Health Organization (WHO) is the reduction or elimination of morbidity due to schistosomiasis (WHO 2012). As I have already discussed in great detail in chapter 4, to achieve this goal there is a need for tools which accurately assess morbidity and determine the effectiveness of interventions (Bergquist *et al.* 2009; Vennervald & Dunne 2006). Several different biomarkers (proteins or enzymes) measured in blood, serum or plasma have been suggested as potentially useful diagnostic and prognostic indicators of underlying pathological conditions in a variety of diseases (Anuradha *et al.* 2012; George *et al.* 2014; Lee *et al.* 2002; Lin *et al.* 2004; Mcsharry *et al.* 1999; WHO 2011*b*; Zakynthinos & Pappa 2009). In schistosome infections, measurement of such markers may prove to be valuable tools for identifying individuals in need of interventions and thereby facilitate timely treatment to prevent the development of severe morbidity (Vennervald & Dunne 2006). As an example, anaemia is usually assessed by measuring the haemoglobin concentration in blood, and the degree of severity with anaemia classified according to WHO guidelines for age and/or gender cut-offs (WHO 2001*b*). Previous studies in schistosomiasis suggest that both iron deficiency and non-iron deficiency anaemia may contribute to low levels of haemoglobin (Coutinho *et al.* 2005; King *et al.* 2005; McGarvey 2000), also discussed in detain elsewhere (Hotez *et al.* 2006). Therefore, in face of these challenges, the WHO also recommends measurement of additional indicators to improve the assessment of anaemia in population surveys (WHO 2005, 2011*c*), such serum ferritin, serum transferrin receptor (s-TfR) and the hormone hepcidin, which regulates iron homeostasis (Ayoya *et al.* 2010; Lee & Beutler 2009). However, the selection and interpretation of the most appropriate indicators for assessing morbidity in large scale population-based studies is complicated by the fact that these biomarkers are non-specific to the type of infection or inflammatory disease condition (Ayoya *et al.* 2010).

In this chapter I sought to address this knowledge gap by evaluating several serum circulating proteins associated with inflammation in children endemically

exposed to schistosome infections. Focusing on several biomarkers instead of a single indicator would aid in the identification of the most informative biomarkers of inflammation that could be used in combination to detect the presence or predict the risk of future morbidity in the field. In particular, I focused on the following serum protein biomarkers applicable under field conditions: Chitinase 3-like 1 protein (CHI3L1), C-reactive protein (CRP), ferritin, resistin, and secretory leukocyte protease inhibitor (SLPI).

CHI3L1 is a known human carbohydrate-binding glycoprotein with no enzymatic chitinase activity secreted by various cell types and is upregulated in a number of human diseases (Kzhyshkowska *et al.* 2007; Lee *et al.* 2011). This protein has been suggested to play an important role in Th2 cytokine-induced inflammation (Mizoguchi 2006). Increased serum levels of CHI3L1 are associated with disease severity and poorer prognosis in organ-specific conditions such as liver fibrosis, inflammatory bowel disease and lung fibrosis (Coffman 2008; Johansen 2006; Kzhyshkowska *et al.* 2007). Recently, elevated serum levels of CHI3L1 have been shown to be associated with presence of haematuria in individuals infected with *S. haematobium* (Appleby *et al.* 2012) and related to hepatic fibrosis due to *S. japonicum* infection (Zheng *et al.* 2005). Although CHI3L1 has been widely used as a prognostic biomarker, there is still no standard reference cut-off for normal serum concentration levels of CHI3L1. Recent attempts to determine reference levels of normal CHI3L1 have been inconclusive, with different studies reporting age-related changes in CHI3L1 levels in healthy individuals over time (Bojesen *et al.* 2011; Korthagen *et al.* 2011).

CRP is a systemic marker of inflammation and tissue damage (Fakanya & Tothill 2014; Macy *et al.* 1997; Ribeiro 1997). It is a non-specific biomarker and its levels in serum have been shown to rise rapidly as a result of tissue damage caused by infection or other acute inflammatory conditions (Fakanya & Tothill 2014). In schistosomiasis, studies have shown that elevated levels of CRP are correlated with presence and/ or severity of morbidity influenced by the inflammatory processes during infection such as iron deficiency anaemia, malnutrition and hepatic fibrosis (Ayoya *et al.* 2010; Coutinho *et al.* 2006, 2005). The normal ranges of CRP vary with the laboratory purposes for which they are measured and low levels of CRP do not always imply that inflammation is not present (Greenland *et al.* 2010).

Ferritin is a major iron storage protein in humans (Wang *et al.* 2010),

and has been widely utilized as a biomarker of iron deficiency anaemia and pathological processes associated with soil-transmitted helminth infections in humans recommended by the WHO for field use (WHO 2001*b*, 2011*b*). It has also been suggested that ferritin arises as a leakage product of damaged cells, and that its serum levels can be rapidly elevated in response to inflammation (Kell & Pretorius 2014; Lee *et al.* 2002; Mcsharry *et al.* 1999). However, its potential as an inflammatory biomarker in schistosome infections has not been widely explored.

Resistin and **SLPI** are other serological biomarkers of inflammation that have also been previously shown to be important in the study of a variety of infections, reflecting disease activity and/or severity (Gipson *et al.* 1999; Jin *et al.* 1997; Lin *et al.* 2004; Nair *et al.* 2006; Silswal *et al.* 2005). Resistin is thought to promote the release of pro-inflammatory cytokines such as IL-6, IL-10 and tumor necrosis factor (TNF)-α, which also play a role in pathogenesis of schistosomiasis (Jang *et al.* 2015; Silswal *et al.* 2005). SLPI is expressed in multiple cell types, and has been implicated in regulating inflammatory processes (Adapala *et al.* 2011; Devoogdt *et al.* 2004; Gipson *et al.* 1999). Knowledge about the expression of SLPI in helminth infections is still lacking, although it has been suggested in other diseases that its levels are upregulated during granulomatous inflammatory responses (Ohlsson *et al.* 2003).

The host immune response to schistosome infection induces the secretion of cytokines whose levels in plasma are important for the aetiology and regulation of inflammation (Caldas *et al.* 2008). These immune responses involve an interplay between Th1, Th2 and T regulatory (Treg) cells, and the cytokines they produce (Mishra *et al.* 2014; Nausch *et al.* 2011). The Th1 acute pro-inflammatory response to schistosome antigens is characterized by the production of IFN-γ (Mwatha *et al.* 1998), which promotes activation of macrophages (Warrington *et al.* 2011) and secretion of other cytokines such as TNF-α and IL-6 (Nair *et al.* 2006; Pearce & MacDonald 2002). The schistosome egg antigens invoke Th2 responses, inducing the release of the cytokines IL-4, IL-5 and IL-13 (Caldas *et al.* 2008; Colley *et al.* 2014; Pearce & MacDonald 2002). These Th2 cytokines have been associated with granuloma formation (Kaplan *et al.* 1998), also reviewed in detail by (Fairfax *et al.* 2012). Previous immunoepidemiological and experimental studies have shown that IL-10 plays a regulatory role in schistosome infections by modulating the effects of Th1 and Th2 immune responses, and thereby preventing damage to the host's tissues (Corrêa-Oliveira *et al.* 1998; Hoffmann *et al.* 2000;

Magalhães *et al.* 2004; Mutapi *et al.* 2007; Redpath *et al.* 2013; Smith & Maizels 2014), also reviewed in detail by Maizels & Yazdanbakhsh (2003). The immune phenotype arising from the balance of these cytokines can be informative of the risk of current and future morbidity associated with schistosome infection. However, the growing number of different markers of morbidity and inflammation still remain to be concurrently validated in preschool children (typically less than 5 years old).

6.1.1 Study aims

The aim of this chapter is to evaluate the utility of the five serological inflammatory biomarkers (CHI3L1, CRP, ferritin, resistin, and SLPI) as indicators of the risk of having schistosome-related morbidity. In addition, I sought to investigate the effect of praziquantel treatment on the circulating levels of the inflammatory biomarkers measured in children naturally exposed to *S. haematobium*. To enhance understanding of the morbidity profile due to schistosomiasis in children, these inflammatory biomarkers will be related to the point-of-care markers of schistosome-related morbidity (microhaematuria, proteinuria and albuminuria) identified and evaluated in chapters 4 and 5, and the cytokines (IFN-γ, IL-4, IL-5, IL-10 and IL-13), that have been previously evaluated and shown in other studies to play a potential role in schistosome immunopathology (Magalhães *et al.* 2004; Milner *et al.* 2010; Mutapi *et al.* 2007; Silveira *et al.* 2004). Establishment of these relationships would elucidate the importance of the inflammatory biomarkers as parameters for the assessment of schistosome-related morbidity.

6.2 Hypotheses

Based on the analysis of the five serum circulating inflammatory biomarkers (CHI3L1, CRP, ferritin, resistin, SLPI), in relation to *S. haematobium* infection levels, evaluated clinical markers of schistosome-related morbidity (microhaematuria, proteinuria and albuminuria) and a set of systemic cytokines (IL-4, IL-5, IL-10, IL-13, IFN-γ), the objective of the current chapter was to test the following null hypotheses:

1. There is no association between circulating levels of inflammatory biomark-

ers and schistosome-related morbidity markers, and the set of systemic cytokines responses.

2. Curative treatment with praziquantel has no effect on circulating levels of the inflammatory biomarkers identified as valuable parameters for the assessment of schistosome-related morbidity.

6.3 Methods

6.3.1 Study population

Children who met the eligibility criteria outlined in section 2.3.4 (chapter 2) and had provided blood samples for serological tests were enrolled into the current study. A total of 346 participants fulfilled the selection criteria and were included in the study cohort for the final analyses as shown in Table 6.1. Of these children, 136 (39.3%) were aged 1–5 years old and 210 (60.7%) were aged 6–10 years old. Participants were further categorized into four age groups following the epidemiological age-prevalence curves for schistosome infections (Woolhouse 1998), as well as considering the balance of sample sizes between the different age groups (Table 6.1). Stool and urine specimens were collected from each participant as already described in section 2.3 of chapter 2. Following parasitology and blood sample collection, children were offered treatment with the standard dose of praziquantel (40 mg/kg body weight).

Table 6.1: Baseline demographic characteristics of the study population.

Variable	Number	Percentage (%)
Sex:		
Male	160	46.2
Female	186	53.8
Age group:		
1–3 years	37	10.7
4–5 years	99	28.6
6–7 years	90	26.0
8–10 years	120	34.7
Total	346	100.0

6.3.2 Determination of infection and morbidity

In chapter 3, I demonstrated the advantages of using the serological technique in addition to the parasitological method in order to improve infection diagnostic accuracy. Thus, in the current chapter, infection with *S. haematobium* was defined as the presence of eggs in urine and/or positive IgM antibody response against soluble egg antigens. The findings in chapter 4 showed that the urinary markers identifying the largest proportion of morbidity attributable to *S. haematobium* infection were albuminuria, microhaematuria and proteinuria. Therefore, these three morbidity markers were chosen for investigation in the current chapter to establish their relationship with the serological inflammatory biomarkers.

6.3.3 Assessment of inflammatory markers

Serum levels of the inflammatory biomarkers (CHI3L1, CRP, ferritin, resistin, SLPI) as well as plasma levels of the cytokines (IL-4, IL-5, IL-10, IL-13, and IFN-γ) were measured by ELISA as previously described in chapter 2 (see section 2.5). Responders were defined as children with sample concentration levels of biomarkers above 0 ng/mL (after subtraction of the blank control), while those children with 0 ng/mL or below were designated as having no detectable levels of the serum inflammatory biomarkers or plasma cytokine responses.

6.3.4 Assessment of praziquantel treatment effect

The treatment effect study focused on those inflammatory biomarkers differing significantly in their levels by schistosome infection status (infected *vs.* uninfected) and/or presence *vs.* absence of point-of-care markers of schistosome-related morbidity. To be included in the post-treatment study, children fulfilling the study selection criteria had to be confirmed egg negative 12 weeks after treatment, if they had received the recommended dose of praziquantel (PZQ). As detailed earlier, there was a group of children who were either absent from school on treatment days or who would not take Western medication on religious grounds but were willing to take part in the study. These effectively became untreated controls who were also included in the 12 month post-treatment survey (Table 6.2). All the study participants were not re-infected at 12 months, as determined by parasitological examinations for eggs in urine.

Table 6.2: Characteristic of participants selected for the treatment study. Levels of the serological biomarkers of inflammation were assessed 12 months after treatment with a single dose of praziquantel (treated, n=120; untreated control, n=23).

Variable	Untreated controls	PZQ treated	Total
Sex, n (%)			
Male	9 (391.)	55 (45.8)	64 (44.8)
Female	14 (60.9)	65 (54.2)	79 (55.2)
Age (years)			
Mean (SD)	6.2 (2.63)	7.9 (1.98)	7.6 (2.18)
Age group, n(%)			
1–3 years	3 (13.0)	1 (0.8)	4 (2.8)
4–5 years	7 (30.4)	14 (11.7)	21 (14.7)
6–7 years	4 (17.4)	32 (26.7)	36 (25.2)
8–10 years	9 (39.1)	73 (60.8)	82 (57.3)

6.3.5 Statistical analyses

The empirical distributions of the inflammatory biomarkers and cytokine responses were explored by means of histograms (Appendix F.1). In order to meet the assumptions of the parametric statistical tests and reduce skewness, data for infection intensity and all the cytokines and inflammatory biomarkers were log-transformed: $\log_{10}(x + 1)$, ascertained using the Box-Cox power transformations method (Box & Cox 1964). For the formulation and results of the Box-Cox transformation model, see Appendix F.2. I assessed the bivariate correlation between inflammatory biomarkers and cytokines, as well as infection intensity using Spearman's rank test, partialling out the effects of age. I used the non-parametric Wilcoxon Signed Rank Test to compare changes in the levels of inflammatory markers post-treatment as the sample sizes for the different subgroups were too small to reasonably assume normality. This non-parametric approach was preferred as it takes into account the magnitude of the observed differences between paired samples (Sprent & Smeeton 2001).

6.3.6 Assessing factors associated with levels of inflammatory biomarkers

I used stepwise linear regression analysis to determine the relations between biomarkers of inflammation, cytokines, infection status and markers of schistosome-related morbidity, sequentially allowing for the effects of sex and age group. The main effects general linear regression model was expressed as follows:

$$Y_i = \beta_0 + \beta_1 \text{Sex} + \beta_2 \text{Age} + \beta_3 \text{Infection status} + \beta_4 \text{Morbidity marker} + \varepsilon_i \quad (6.1)$$

where:
Y_i is the log-transformed response (inflammatory biomarker or cytokine) variable
β_0 is the intercept term
$\beta_1, \ldots, \beta_4$ are parameters indicating the main effects of the predictor variables
ε_i is a random error term, indicating the measurement error in the response variable

The model-building process involved forward stepwise inclusion of the covariate terms and their two-way interactions. In this analysis, the forward stepwise procedure was preferred over the backward elimination method for two main reasons: 1) the main effects model was used as the baseline model, hence there was no potential of inflated mean square errors (MSEs) in model building because of omitted important predictors; 2) since the number of possible interactions between the potential confounding predictors was moderately large, by principle of parsimony (Stone 1974), a saturated interactions model would compromise the consistency and efficiency of model parameter estimations (Gould & Lawless 1988). The MSEs were used to compare the improvement in models with each of the interaction predictor variables included. Based on this criterion, a model with the smallest MSE was deemed the best model. Prior to making inferences based on final models, Q-Q plots of the residuals were used to check for normality and scatterplots of residuals on predicted values of each of the response variables were used to check for constance of variance. In addition, the Brown-Forsythe test was used to formally validate the homogeneity of variance assumption (Kutner *et al.* 2005).

During the model building process, inclusion of the interaction terms in

stepwise regression analyses did not result in marked improvement of the main effects models, assessed by comparing the mean square errors (see Appendix F.4). Furthermore, since these two-way interactions were not statistically significant (P>0.05), I limited all the interpretations of the findings to main predictor effects only.

6.3.7 Assessing multivariate relations between inflammatory biomarkers

Canonical Correlation Analysis

The method of canonical correlation analysis (CCA) was used to investigate how the set of serum circulating inflammatory biomarker variables related to the set of cytokine responses. Briefly, the aim of canonical correlation analysis is to measure the correlation between two sets of variables and is suitable for situations where there are multiple inter-correlated outcomes. This is done by finding pairs of linear combinations that are maximally correlated with one another, then, it determines a pair that has the largest correlation among all pairs (Johnson & Wichern 2007). The strength of the overall relationship between two sets of variables is measured by the canonical correlation coefficient (R), which represents the bivariate correlation between the two canonical variates from the different sets of variables. The absolute values of canonical coefficient loadings reflect the contribution of each of the individual variables to the explained variation between the two sets of variables (Johnson & Wichern 2007). In this current chapter, I used the canonical loadings to explore the relative importance of each of the cytokine responses to the inflammatory environment represented by serum circulating levels of the protein biomarkers, adjusted for the effect of age. The significance of the bivariate correlation was tested using the Wilks' lambda (Λ) multivariate statistic.

Principal Component Analysis

To determine the multivariate relationships among the set of inflammatory variables and explain the underlying variability structure associated with each group of inflammatory markers, a principal component analysis (PCA) was carried out. Principal components (PCs) with eigenvalues greater than 1 were extracted and variables with absolute factor loadings greater than or equal to 0.5 were interpreted (Johnson & Wichern 2007). The extracted PCs were then included in linear regression models to test for the relationship between schistosome-related morbidity markers and subgroups of biomarkers of inflammation, adjusted for sex, age group, and infection status/intensity as outlined above.

6.4 Results

6.4.1 Schistosome infection levels

The prevalence and intensity of *S. haematobium* infection increased with age, and the highest levels were observed between the ages of 8–10 years old as shown in Table 6.3. Based on the WHO categorization of *S. haematobium* infection intensity burden, it was noted that 5.7% (n=19) of the children carried heavy infection intensities (mean=217.5 eggs/10 mL urine; range=58.3–1013.0 eggs/10 mL eggs) and 23.2% (n=77) of the children carried light infections (mean=11.6 eggs/10 mL urine; range=0.3–48.0 eggs/10 mL eggs).

Table 6.3: Prevalence and intensity of *S. haematobium* infection by age group.

		Mean (minimum-maximum)		Infection prevalence (95% CI)	
Age group	**N**	**Egg count**	**SEA IgM**	**Parasitology**	**Parasitology + Serology**
1–3 years	37	2.0 (0.0–2.0)	0.35 (0.02–1.22)	3.0 (0.0–9.2)	18.9 (5.7–32.2)
4–5 years	99	8.6 (0.0–30.5)	0.36 (0.03–1.01)	9.6 (3.5–15.6)	27.3 (18.3–36.2)
6–7 years	90	17.1 (0.0–1000)	0.58 (0.01–1.28)	25.8 (16.6–35.1)	63.3 (53.2–73.5)
8–10 years	120	29.5 (0.0–1013)	0.68 (0.09–1.17)	54.3 (45.1–63.5)	80.0 (72.7–87.3)
Overall	346	15.1 (0.0–1013)	0.53 (0.01–1.28)	28.9 (24.0–33.8)	54.0 (48.8–59.3)

Infection intensity determined by parasitological egg count/10 mL urine.
IgM SEA mean Optical density levels measured at 405 nm for the serological diagnosis of infection.
Combined infection prevalence=Parasitology + Serology.

6.4.2 Detectable levels of inflammatory biomarkers and cytokines

The histograms to explore the distribution of the inflammatory biomarkers and cytokines indicated that there were some children who expressed elevated systemic levels of these markers, while some children showed low levels (Figure F.1). Of the assayed serum samples, more than 90% of the children produced detectable levels of inflammatory protein biomarkers (see Figure F.2). The percentage of plasma samples with detectable amounts of IL-10 and IL-13 was significantly higher (P<0.001; see Figure F.2) in infected children compared to uninfected children. Similar results were obtained when comparisons were made between *S. haematobium* egg-positive and egg-negative children (Table F.5).

6.4.3 Levels of inflammatory biomarkers by age

Exploratory profile plots showed different trends of inflammatory biomarkers in relation to age as illustrated in Figure 6.1. Without formally testing at this stage, a slightly increasing trend with age among infected children was observed for for all the inflammatory biomarkers except for CHI3L1, which seemed to drop with age (Figure 6.1). Plasma levels of the cytokines also showed different patterns with age between *S. haematobium* infected and uninfected children as illustrated in Figure 6.1. However, the observed overall mean change in concentration levels was relatively small in terms of magnitude for both inflammatory biomarkers and cytokine responses.

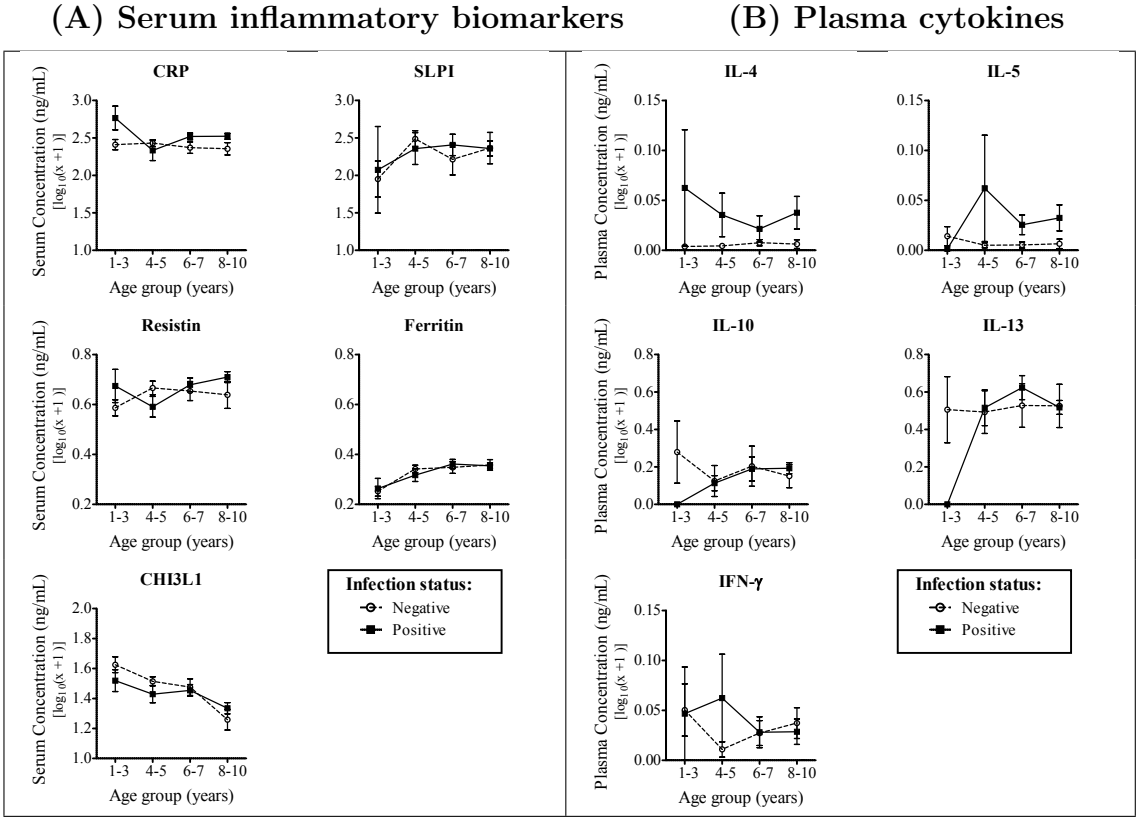


Figure 6.1: Age profiles of inflammatory biomarkers and systemic cytokine responses. Mean circulating levels by *S. haematobium* infection status determined by parasitological and serological diagnostic techniques. Error bars indicate the standard error of the mean.

6.4.4 Levels of inflammatory biomarkers by infection intensity

Plots to explore the relationship between *S. haematobium* infection intensity and levels of biomarkers are shown in Figure 6.2. The infection intensity determined by egg examination in urine was not correlated with serum levels of the inflammatory biomarkers or cytokine responses, partialling out the age effects (Figure 6.2). The raw SEA IgM antibody titres significantly increased with serum levels of CRP (r=0.15; P=0.005) and plasma levels of IL-4 (r=0.17; P=0.017) but not with the other biomarkers studied.

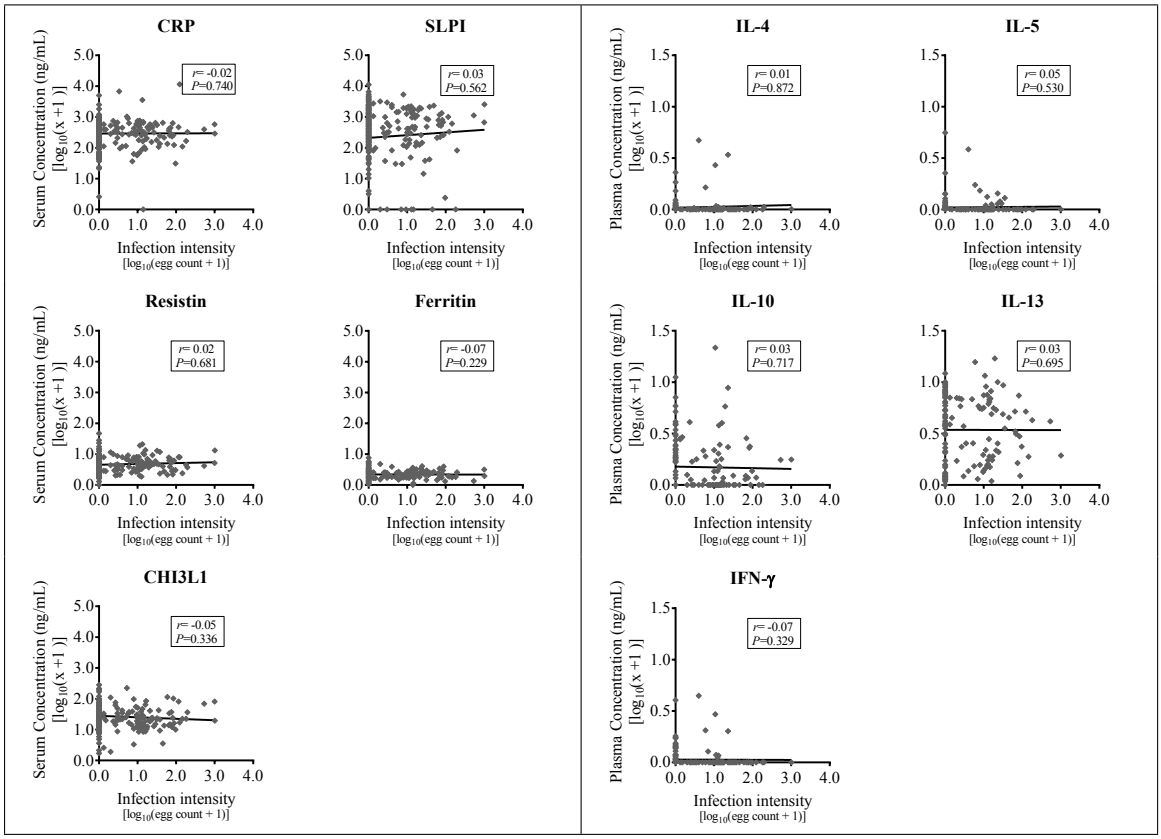


Figure 6.2: Individual distribution of *S. haematobium* infection intensity by levels of inflammatory biomarkers and cytokine responses. Correlation between infection intensity and inflammatory biomarkers and cytokines determined by Spearman's rank test, adjusted for age.

6.4.5 Correlations between inflammatory biomarkers and cytokines

Assessing the bivariate relations between the inflammatory biomarkers, levels of CRP showed the strongest positive correlation with CHI3L1 (Table 6.4). In addition, circulating levels of CRP were positively associated with the pro-inflammatory cytokine, IL-13. Furthermore, IL-10, a regulatory cytokine was also significantly correlated with levels of CRP and all the other pro-inflammatory cytokines as shown in Table 6.4.

The set of inflammatory biomarkers correlated significantly with the set of cytokine variables as shown by the results of the canonical correlation analysis

Table 6.4: Correlations between inflammatory biomarkers and plasma cytokine responses, partialling out the effect of age. Spearman's rank correlations. *P*-values are shown in parentheses (). Significant[a] associations are highlighted and in bold.

Variable	CHI3L1	CRP	Ferritin	Resistin	SLPI	IL-4	IL-5	IL-10	IL-13	IFN-γ
CRP	**0.20 (<0.001)**									
Ferritin	0.02 (0.738)	**0.15 (0.008)**								
Resistin	0.05 (0.379)	**0.17 (0.002)**	**0.18 (0.001)**							
SLPI	0.05 (0.399)	-0.02 (0.751)	**0.21 (<0.001)**	**0.27 (<0.001)**						
IL-4	0.01 (0.907)	0.03 (0.682)	0.10 (0.165)	0.11 (0.124)	**0.29 (<0.001)**					
IL-5	0.08 (0.287)	-0.01 (0.874)	**0.32 (<0.001)**	**0.29 (<0.001)**	**0.33 (<0.001)**	**0.24 (<0.001)**				
IL-10	0.07 (0.444)	**0.21 (0.014)**	**0.21 (0.012)**	0.06 (0.496)	**0.28 (0.001)**	**0.30 (0.002)**	**0.24 (0.017)**			
IL-13	0.01 (0.932)	**0.22 (0.008)**	0.10 (0.260)	0.10 (0.251)	**0.29 (<0.001)**	0.18 (0.084)	0.08 (0.400)	**0.53 (<0.001)**		
IFN-γ	-0.03 (0.636)	0.05 (0.456)	**0.23 (0.001)**	0.01 (0.897)	**0.26 (<0.001)**	**0.50 (<0.001)**	**0.40 (<0.001)**	**0.42 (<0.001)**	**0.31 (0.002)**	

[a] *P*-values not corrected for family-wise error.

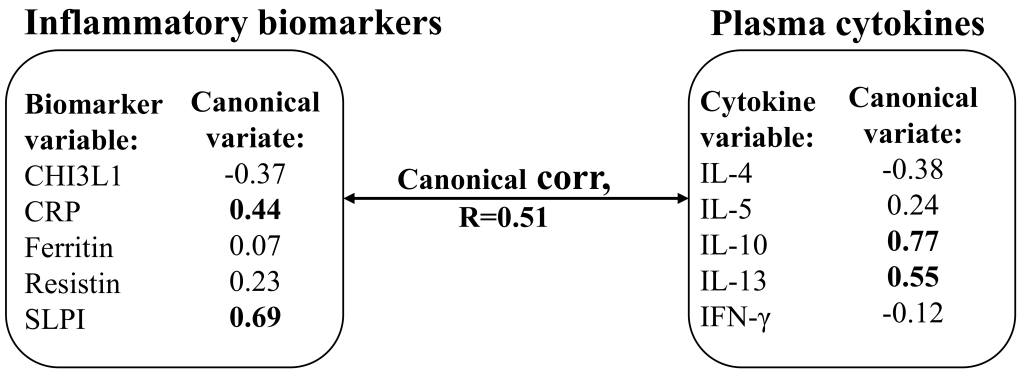


Figure 6.3: Standardized canonical variate coefficients and canonical correlation (R) analysis results. Canonical correlation analysis to identify and quantify the multivariate association between the set of inflammatory biomarkers and the cytokine responses. The absolute magnitudes of the canonical covariate coefficients give the contributions of the individual biomarker or cytokine response to the the corresponding canonical variable.

in Figure 6.3. As was observed in bivariate correlations (Table 6.4), CRP was identified, together with SLPI as the inflammatory biomarkers contributing most to the observed variation in the inflammatory processes observed in this population (Figure 6.3). Among the cytokines, IL-10 and IL-13 contributed the most to the inflammatory processes observed in this population (Figure 6.3). Additional canonical correlation analysis to investigate whether the set of inflammatory biomarkers could be related to the set of schistosome-related morbidity markers and cytokine responses simultaneously revealed a positive, but non-significant multivariate association (R=0.29; Wilk's Λ=0.94; F=1.03; P=0.422).

6.4.6 Clustering of inflammatory biomarkers

Table 6.5 summarizes the principal components analysis results to determine multivariate relations between groups of inflammatory biomarkers and cytokines. There was a reduction in the set of the original five inflammatory variables to two distinct composite clusters, explaining 89.3% of the total variability in the data (Table 6.5). CHI3L1 and CRP stood in a separate component, both with strong positive factor loading coefficients. The rest of the biomarkers; ferritin, resistin, and SLPI had a similar contribution to the other resulting component of the PCA (Table 6.5). The pro-inflammatory cytokine IL-13 had the strongest contribution

to the resulting components compared to other cytokines as indicated by its high positive factor loading coefficient and belonged to a separate component to the rest of the cytokines. The results showed that the cytokines IL-4, IL-10 and IFN-γ had similar contributions to the other resulting separate principal component (Table 6.5). Further sub-group analysis on *S. haematobium* egg-positive children to explore the inflammatory environment suggested by the above results revealed that CHI3L1 and CRP had the strongest contributions compared to the other biomarkers, with factor loadings 0.73 and 0.64, respectively.

Table 6.5: Principal Components Analysis (PCA) factor loadings of the inflammatory biomarkers and plasma cytokine responses. The PCA were conducted separately for the inflammatory biomarkers and cytokines. Strong factor loadings in absolute magnitude (≥0.5) are highlighted and indicated in bold.

Variable	Principal Component Loadings	
	PC1	PC2
Inflammatory biomarkers:		
% of variation explained	**76.8%**	**12.5%**
CHI3L1	0.12	**0.75**
CRP	0.37	**0.57**
Ferritin	**0.55**	-0.24
Resistin	**0.57**	-0.11
SLPI	**0.50**	-0.22
Plasma cytokine responses:		
% of variation explained	**71.7%**	**22.9%**
IL-4	**0.52**	-0.22
IL-5	0.42	-0.39
IL-10	**0.50**	0.32
IL-13	0.27	**0.82**
IFN-γ	**0.50**	-0.18

The principal components were extracted according to the proportion of variation in the study population they explained (i.e., at least ≥80%).

6.4.7 Relations between inflammatory biomarkers and morbidity markers

In the regression analyses to determine the relationship between the biomarkers of inflammation and markers of schistosome-related morbidity, only ferritin showed a significant association with proteinuria (Table 6.6). However, the pairwise comparisons revealed that the mean levels of this biomarker were significantly lower in proteinuria positive children (mean=1.17 ng/mL; SE=0.074) compared to children negative for proteinuria (mean=1.34 ng/mL; SE=0.051). The adjusted mean serum levels of the inflammatory biomarkers by presence/absence of markers of schistosome-related morbidity are illustrated in Figure 6.4 to aid the interpretation of these results. Furthermore, these results were consistent with those from subgroup analyses focusing on *S. haematobium* egg-positive children only (see Table F.6). The extracted principal components did not reveal significant associations with any of the three urinary markers of schistosome-related, after sequentially allowing for the effects of sex, age and infection status (Table 6.6). Among the cytokine responses (see Table F.4), only IL-4 sowed a significant (post-hoc t=2.2; P=0.027), but negative association with presence of proteinuria: negative, mean IL-4=0.11 ng/mL (SE=0.040) *vs.* positive, mean IL-4=0.01 ng/mL (SE=0.013).

Table 6.6: Results of regression analysis to determine the relationship between inflammatory biomarkers and schistosome-related morbidity. The relationship was assessed after allowing for the effects of host factors sex, age group and infection status determined by a combination of parasitology and serology. Association with schistosome-related morbidity markers: (A) Microhaematuria, (B) Proteinuria and (C) Albuminuria. Significant P-values are highlighted and shown in bold.

	CHI3L1		**CRP**		**Ferritin**		**Resistin**		**SLPI**		**PC1**[a]		**PC2**[b]	
Variable	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*
(A)														
Sex	**7.50**	**0.007**	0.79	0.374	0.08	0.773	0.42	0.518	1.10	0.296	1.69	0.195	**4.25**	**0.040**
Age group	**8.39**	**<0.001**	1.91	0.128	**4.76**	**0.003**	2.34	0.074	1.04	0.377	**2.91**	**0.035**	**5.66**	**0.001**
Infection status	0.46	0.500	**4.25**	**0.040**	0.13	0.720	0.18	0.671	0.02	0.901	0.38	0.537	0.45	0.505
Microhaematuria	0.28	0.597	2.47	0.117	0.12	0.732	0.32	0.571	2.91	0.089	0.37	0.543	0.99	0.320
(B)														
Sex	**7.65**	**0.006**	0.96	0.328	0.05	0.821	0.30	0.583	1.16	0.282	1.62	0.204	**4.63**	**0.032**
Age group	**8.54**	**<0.001**	1.80	0.148	**5.24**	**0.002**	2.57	0.055	0.92	0.430	**3.06**	**0.029**	**6.08**	**0.001**
Infection status	0.43	0.514	**3.98**	**0.047**	0.12	0.727	0.22	0.640	0.01	0.918	0.39	0.534	0.42	0.518
Proteinuria	0.08	0.771	0.43	0.513	**4.91**	**0.027**	0.07	0.797	0.11	0.742	2.03	0.156	0.00	0.996
(C)														
Sex	3.68	0.056	0.02	0.887	0.11	0.7365	0.04	0.842	1.14	0.287	0.43	0.510	1.26	0.262
Age group	**5.63**	**0.001**	0.98	0.401	**4.60**	**0.004**	2.13	0.096	1.92	0.126	**3.47**	**0.017**	**5.70**	**0.001**
Infection status	0.30	0.584	**4.62**	**0.033**	0.01	0.943	0.59	0.443	0.09	0.763	1.17	0.281	0.37	0.546
Albuminuria	0.02	0.891	0.18	0.675	0.49	0.485	0.09	0.761	1.92	0.167	0.01	0.944	0.22	0.639

Children were classified as infected if egg positive and/or serologically positive using SEA IgM antibody levels
[a] *PC1 (ferritin; resistin; SLPI).*
[b] *PC2 (CHI3L1; CRP).*

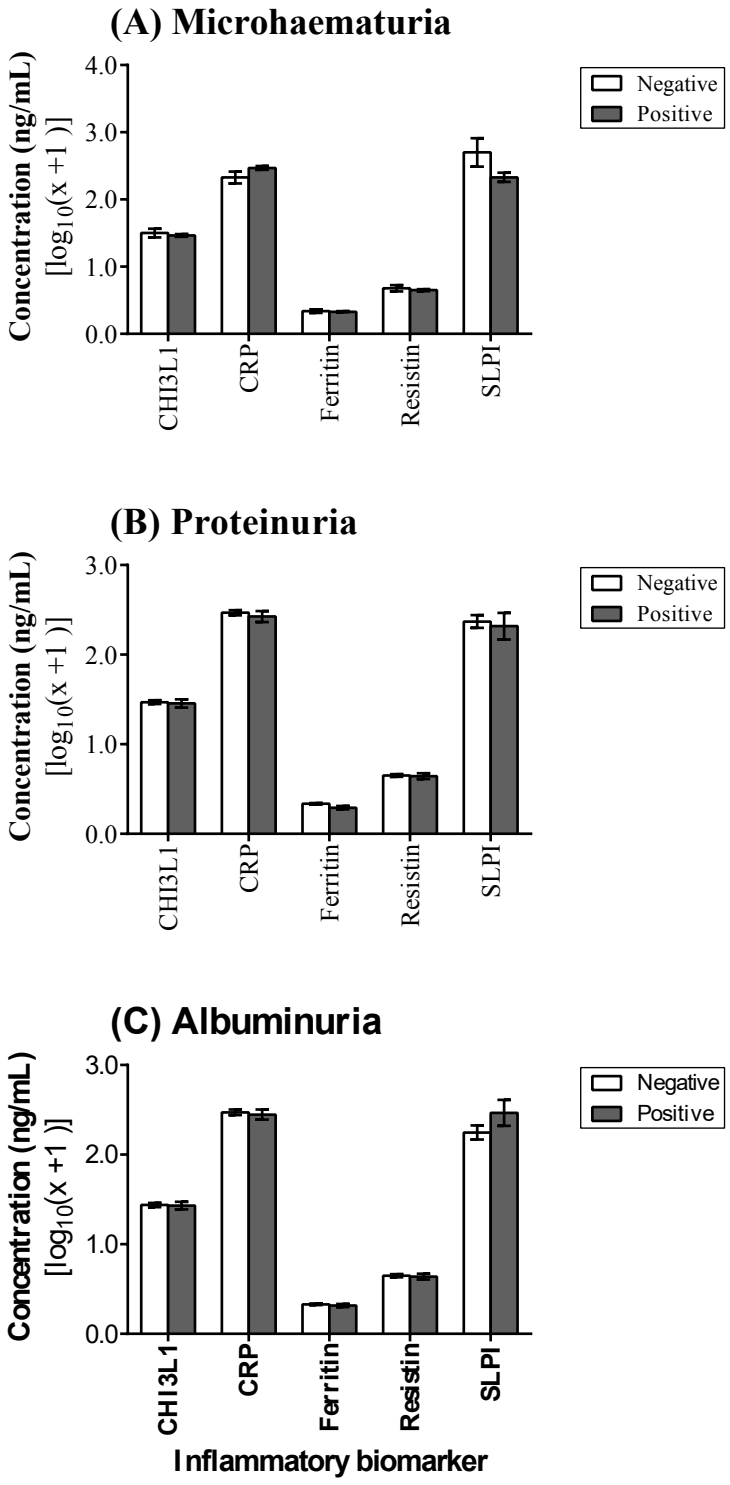


Figure 6.4: Adjusted mean levels of inflammatory biomarkers by presence/absence of schistosome-related morbidity markers. The means were adjusted for sex, age group and baseline infection status determined by a combination of parasitological and serological techniques.

6.5 Effect of praziquantel treatment

To address the research question of whether praziquantel had an effect on the circulating inflammatory biomarkers, changes in the levels of CHI3L1 and CRP were assessed 12 months after treatment (see Table 6.2). The two biomarkers were selected based on the fact that they showed strong contribution to the inflammatory clustering in principal components analysis, and when assessed individually or in multivariate analyses they showed significant relations with other biomarkers and pro-inflammatory cytokines or infection status.

6.5.1 Individual changes in levels of CHI3L1 and CRP

Exploring changes in individual levels of the two inflammatory biomarkers, it was observed that of the 120 treated children, CHI3L1 levels decreased in only 9 (7.8%) children (6 of which were infected at baseline) 12 months after treatment. CRP levels dropped in 37.9% of the treated children 12 months after treatment and the majority of these children were also positive for infection at baseline (Figure 6.5). Similar patterns were observed when considering a subgroup of children egg-positive at baseline (see Figure F.3). Individual serum levels of CHI3L1 increased after 12 months in all untreated children who were infected at baseline, except in one child whose levels remained unchanged (Figure 6.5). However, CRP levels remained unchanged at 12 months in most of the untreated children (Figure 6.5).

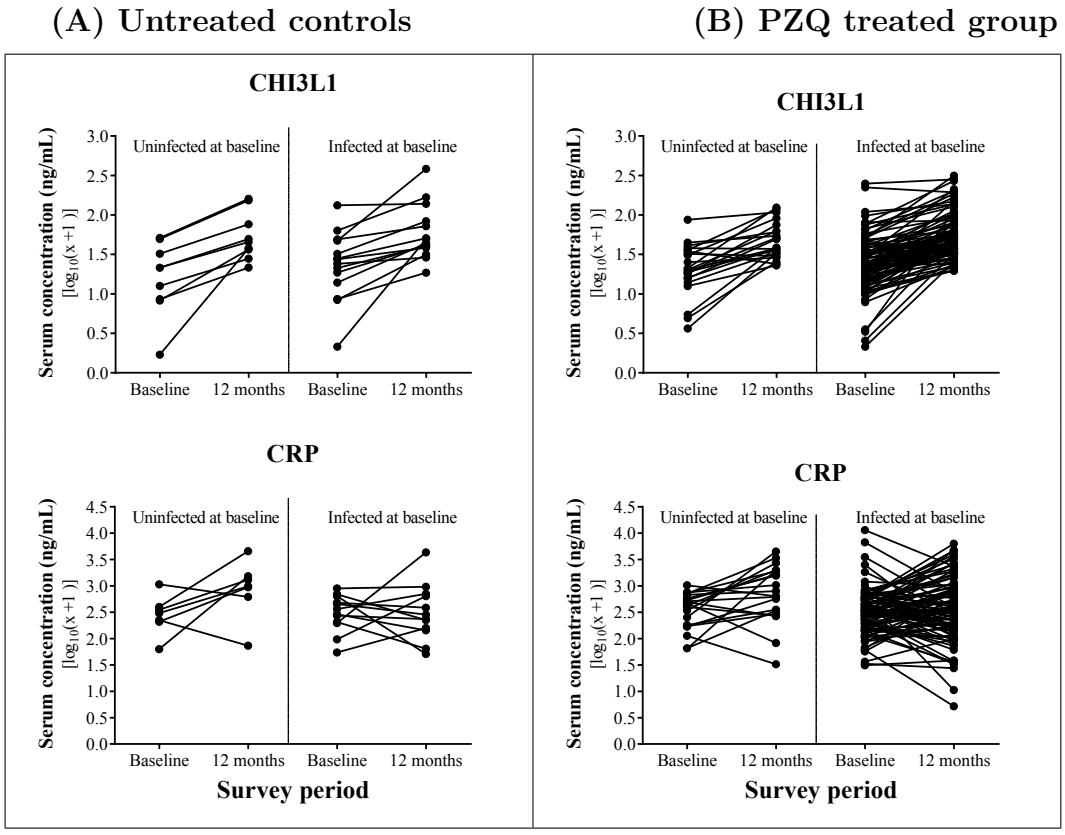


Figure 6.5: Individual serum levels of CHI3L1 and CRP levels at baseline and 12 months after treatment split by baseline infection status determined by parasitology and serology. Changes in circulating levels of inflammatory biomarkers in treated (n=120) and untreated (n=23) children.

6.5.2 Effect of treatment on mean levels of inflammatory biomarkers

Pre-treatment and 12 months post-treatment mean levels of the two inflammatory biomarkers by baseline infection status are shown in Table 6.7. Levels of CHI3L1 increased significantly from baseline in both treated and untreated children 12 months post-treatment. However, when comparing the differences in post-treatment mean changes in CHI3L1 among children diagnosed positive for infection at baseline, no significant differences were observed between the treated and untreated. On the other hand, PZQ treatment did not result in a significant change in levels of CRP 12 months after treatment (Table 6.7). Furthermore, the results of the subgroup analysis conducted in children found *S. haematobium* egg-positive at baseline also revealed similar findings, showing a significant increase in CHI3L1 in treated children at 12 months (Figure F.3).

Table 6.7: Effect of praziquantel on serum levels of CHI3L1 and CRP. Comparison of changes in log-transformed levels of inflammatory biomarkers 12 months post-treatment, split by baseline infection status determined by both parasitological and serological diagnostic techniques. Test-statistic P-values are from the Wilcoxon Signed Rank Test.

	Serum CHI3L1 levels (ng/mL)					
	Baseline		**12 months**		**Difference**	
Group	**Mean±SE**	**Median**	**Mean±SE**	**Median**	**(95% CI)**	$\boldsymbol{P}^{1}$
Untreated						
Negative (n=9)	1.19±0.16	1.33	1.72±0.10	1.66	0.53 (0.28–0.78)	**0.004***
Positive (n=14)	1.36±0.12	1.41	1.77±0.09	1.67	0.41 (0.21–0.62)	**<0.001***
PZQ treated						
Negative (n=22)	1.30±0.07	1.30	1.67±0.05	1.58	0.37 (0.24–0.54)	**<0.001***
Positive (n=98)	1.37±0.03	1.38	1.73±0.03	1.69	0.36 (0.30–0.41)	**<0.001***
	Serum CRP levels (ng/mL)					
Untreated						
Negative (n=7)	2.45±0.13	2.48	2.94±0.21	2.99	0.49 (-0.11–1.11)	0.078
Positive (n=12)	2.48±0.15	2.51	2.49±0.15	2.41	0.02 (-0.40–0.43)	0.791
PZQ treated						
Negative (n=20)	2.53±0.08	2.65	2.85±0.12	2.85	0.32 (0.06–0.59)	**0.027**
Positive (n=93)	2.50±0.05	2.51	2.57±0.06	2.54	0.07 (-0.06–0.19)	0.171

SE= standard error of the mean.

[1]Bonferroni corrected nominal level of significance for 4 pairwise-comparisons for each of the biomarkers was set at: $\alpha/4$=0.0125.

[*]Significant at Boniferroni corrected nominal level.

6.6 Discussion

In the preceding two chapters, I have demonstrated that subtle morbidity due to *S. haematobium* infection does occur in young children and that the current point-of-care markers of schistosome-related morbidity can be effectively reduced by a single dose of antihelminthic treatment with praziquantel. Identification of additional biomarkers that can be used under field conditions as early indicators of underlying pathological changes and clinical outcomes related to schistosomiasis in children would provide an invaluable tool for the design of improved control programmes. In addition, these inflammatory responses can be targeted for early treatment, thereby preventing development of chronic disease in the later years of life (Bhargava *et al.* 2003; Vennervald & Dunne 2006). In recent years, a growing number of inflammatory mediators have been investigated as indicators of disease progression and severity (Ayoya *et al.* 2010; Ong'echa *et al.* 2011). However, the manifold serum circulating biomarkers are non-specific (Knopp *et al.* 2013), hence their utility as non-invasive tools to diagnose underlying pathology associated with schistosomiasis in these young children still needs to be thoroughly validated.

In the current chapter, I examined five serum circulating proteins associated with the inflammatory processes during diverse health conditions in order to evaluate their potential as indicators of current morbidity and/or prognostic markers of future *S. haematobium*-related morbidity in a single population of children aged 1–10 years. This study is among the first to simultaneously evaluate multiple inflammatory biomarkers in a single population of young children naturally exposed to *S. haematobium*. As such, it adds data to the understanding of the factors associated with pathogenesis of paediatric schistosomiasis.

The varying age patterns in levels of infection and inflammatory responses observed in the current study are consistent with reports from other epidemiological studies of human schistosomiasis, reflecting an accumulation of worm burden and increased exposure to the parasite antigens as children grow older and make more frequent independent contacts with infective waters (Mitchell *et al.* 2011; Mutapi *et al.* 2011; Stothard *et al.* 2011). In the present study, I used both parasitological and serological methods to detect infection. On one hand, it could be argued that only children excreting eggs in urine matter in the investigation of inflammatory processes in this current study. Yet on the other hand, the standard parasitology egg count misses active infections as previously noted (Berhe *et al.* 2004; De Vlas

et al. 1997). Furthermore, its limitations in detecting infections in young children demonstrated in my findings in chapter 3, justify the use of the more sensitive serological method as an additional diagnostic tool. Nonetheless, as an extra step, I further confirmed the existence of an inflammatory environment among the endemically exposed children by performing subgroup analysis on egg-positive children only which resulted in similar findings to those observed in children diagnosed by both parasitology and serology (see Appendix F).

In my study, a high proportion of infected children had detectable plasma levels of IL-10 and IL-13, suggestive of an underlying immune-mediated inflammatory environment in this population. These data also support previous findings in which the percentage detectable cytokine levels were significantly associated with presence of parasite eggs in stool or urine (De Souza, Robson Da Paixão *et al.* 2012; Imai *et al.* 2011; Magalhães *et al.* 2004; Milner *et al.* 2010; Mutapi *et al.* 2007; Silveira *et al.* 2004). However, in my study the percentage with detectable levels of inflammatory biomarkers was high in both infected and uninfected children (as detected either by egg status only or using the combination of both parasitology and serology methods). This finding may be plausibly be due to an increase in background levels of these biomarkers influenced by other non-schistosome related agents such as *Giardia*, *Plasmodium* antigens and house dust mite, also commonly reported among rural Zimbabwean populations (Imai *et al.* 2011; Rujeni *et al.* 2013).

The inflammatory environment is characterized by a complex range of different responses rather than a single immune response marker (Bourke *et al.* 2012). The inter-correlations of inflammatory mediators during infection further complicates the identification of useful biomarkers of morbidity (Charpentier *et al.* 2013; Ong'echa *et al.* 2011; Zakynthinos & Pappa 2009). In my current study, two inflammatory biomarkers, CHI3L1 and CRP emerged as the most contributors to the underlying inflammatory environment suggested by the results of multivariate analyses. The possibility of existence of an inflammatory environment in these children was further strengthened by the different groupings of the cytokine responses and their correlation with inflammatory biomarkers, in which IL-10 and IL-13 were cited as the major players in the inflammatory process. The involvement of these cytokines as mediators of inflammatory responses associated with schistosome-induced pathology has been reported in previous experimental and human studies (Magalhães *et al.* 2004; Mutapi *et al.* 2007; Silveira *et al.*

2004; Smith & Maizels 2014). However, more research focusing on parasite-specific cytokine responses is needed in order to confirm the current findings and further enhance the understanding of the interactions between these serological biomarkers and cellular immune responses in relation to the risk of morbidity in preschool-aged children.

The levels of the different inflammatory biomarkers and cytokines were not correlated with infection intensity and only a weak association was established between some of these biomarkers with schistosome infection status. In addition, despite the distinct inflammatory profiles revealed by principal component analysis, the results of regression analyses of individual inflammatory biomarkers and cytokines or the extracted components did not reveal any robust associations with the point-of-care (POC) markers of schistosome-related morbidity. In contrast to my findings, Appleby *et al.* (2012) in their study showed that elevated levels of CHI3L1 were significantly positively correlated with presence of haematuria in individuals infected with *S. haematobium*. In a similar fashion, other studies in Zimbabwe and Mali also reported significantly higher levels of serum ferritin and CRP in schistosome infected school children compared to uninfected individuals (Ayoya *et al.* 2010; Friis *et al.* 1996; Reilly *et al.* 2012). However, when comparing the ranges in concentration levels of these indicators of inflammation (CHI3L1, CRP and ferritin) between the reported studies, it was noted that the circulating levels among children in my present study were on the lower end of serum levels observed in the previous studies cited above.

The effect of chemotherapy on circulating inflammatory biomarkers during diverse disorders has produced conflicting reports, and still has yet to be elucidated in the context of schistosomiasis. In my present study, the effect of curative treatment with praziquantel was assessed on CHI3L1 and CRP, the two biomarkers identified as showing the most influence on the inflammatory processes observed in this population. After 12 months of treatment, no changes were observed in levels of CRP, but CHI3L1 levels increased, although these changes were comparable to those of untreated controls. This, however contradicts with results of other studies, which reported a significant reduction in CRP and CHI3L1 levels in schistosome infected individuals following antihelminthic chemotherapy with PZQ (Appleby *et al.* 2012; Coutinho *et al.* 2006).

There are several possible explanations for the results presented here. Firstly, the high percentage of detectable levels of biomarkers and some cytokines in

uninfected children, as well as the lack of robust relationship between these inflammatory biomarkers and schistosome infection levels could be attributed to other unrelated confounding environmental background factors (Lee *et al.* 2008). Changes over time in circulating inflammatory biomarkers arising from other background sources and the ageing process have also been reported in healthy individuals across general populations (Bojesen *et al.* 2011; Bottino *et al.* 2015; Korthagen *et al.* 2011; Prescott 2013; Woods *et al.* 2012). Thus, it could simply be the case that the inflammatory mechanisms observed in these children were elicited by other pathogens since the inflammatory biomarkers are non-specific and the cytokine responses investigated in this study were not exclusively targeted to schistosome infection. Secondly, elevated levels of inflammatory mediators have been associated with poorer prognosis and disease severity such as seen in chronic inflammatory conditions (Libreros *et al.* 2012; Ong'echa *et al.* 2011). Thus, the results from my study showing lack of association of the biomarkers with measures of schistosome-related morbidity could be suggestive of that the levels of these inflammatory biomarkers in this current population of young children who are more likely to have acute infections may be too low to have apparent physiological or biochemical consequences such as those reported in other studies (Appleby *et al.* 2012; Ayoya *et al.* 2010). To support this notion, evidence from a recent study suggested an increased risk of developing bladder morbidity in the second decade of life increased in children chronically infected with *S. haematobium* (Garba *et al.* 2010). More studies involving both preschool children (aged ≤5 years) and older individuals with a longer cumulative history of exposure to schistosome infection would be valuable in elucidating the conflicting evidence about relations between inflammatory biomarkers and POC markers of schistosome-related morbidity.

Lastly, it is unsurprising that antihelminthic treatment with PZQ did not result in a positive reduction in the levels of serum circulating proteins implicated in exacerbating inflammation since no robust relationship between these biomarkers and schistosome infection or related morbidity was observed, as already discussed above. It has been shown in other unrelated studies that the benefits of chemotherapy, effectively resulting in marked changes in circulating levels of inflammatory biomarkers in serum are more pronounced in individuals with chronic or more severe pathology, (discussed in detail by Zakynthinos & Pappa (2009)). Another factor to consider is that some studies on schistosomiasis that reported significant treatment effects had shorter post-treatment follow-ups

of up to 6 weeks (Appleby *et al.* 2012; Coutinho *et al.* 2006). Hence, the 12 months follow-up time at which treatment effects were assessed in the present study might have been too long to show conclusive evidence for treatment related changes in levels of inflammatory biomarkers.

A major strength of my study is that it concurrently investigated several inflammatory biomarkers and cytokine responses to enhance understanding of the relations between these biomarkers. However, the study had some limitations to be acknowledged. Serum circulating biomarkers mainly reflect levels of systemic inflammation, hence other potential confounding factors could influence the current findings. This may limit the applicability of these biomarkers as tools for control. Caution should be taken when extrapolating the results in relation to populations other than of children aged below 10 years of age as this study did not include data on chronic infections associated with older individuals.

6.7 Conclusions

Taken together, the results of this current chapter demonstrated the existence of an underlying inflammatory environment characterised by different sets of biomarkers of inflammation and cytokine responses. The interrelations found in the present study suggest that CRP and CHI3L1 may be useful markers for screening children who are at risk of developing chronic inflammatory conditions. However, the relationship between these biomarkers and schistosome infection, as well as the current point-of-care markers of morbidity was not robust and requires to be explored further to determine their utility as tools for assessing morbidity due to schistosomiasis. The present study revealed no effect of antihelminthic treatment with praziquantel on circulating CRP levels and the magnitude of change in CHI3L1 levels did not differ between treated and untreated children, suggesting their limitation as potential indicators in children for evaluating the impact of interventions in schistosome control programmes.

Chapter 7

General discussion

7.1 Introduction

In this thesis, I focused on urogenital schistosomiasis, an important but neglected infectious disease affecting over 100 million people, mainly in Africa (Gryseels *et al.* 2006). In these endemic areas, urogenital schistosomiasis is associated with high morbidity in children who carry the heaviest burden of infection (Fenwick *et al.* 2009; Gryseels & de Vlas 1996; Hotez & Fenwick 2009). Preschool children (aged ≤5 years old) have previously been neglected both in terms of research and control, which has been further exacerbated by poor diagnosis of infection in the field (also noted by Mutapi (2015)). It not until recently (2010) that the WHO has recommended praziquantel treatment and the inclusion of preschool children in national schistosome control programmes (WHO 2011*b*). However, the burden of disease among these children still remains poorly understood and consequently poorly quantified. Furthermore, the performance of the currently available tools for detecting infection and morbidity is still yet to be thoroughly and systematically evaluated.

I have assessed the utility of currently available methods for diagnosing *S. haematobium* infection (chapter 3) in endemically exposed preschool (1–5 years) and primary school children (6–10 years). Selected point-of-care markers of schistosome-related morbidity applicable under field-based conditions as recommend by the WHO, and several different serological biomarkers of inflammation that could predict early stages of immune-mediated pathology due to schistosomiasis were also investigated in this thesis (chapters 4 and 6). Using a combination of applied statistical methods, I determined the effect of praziquantel

treatment on factors associated with *S. haematobium* infection and morbidity in children aged 1–5 years and the findings were compared with those observed in children aged between 6–10 years old, who are the current targets of the schistosome control programmes (chapter 5).

In this present chapter, the major findings with regard to the research questions (see chapter 2) are summarized. The main conclusions based on the findings of the studies set forth in the preceding chapters of this thesis are outlined. Furthermore, the strengths and limitations of the methods used in thesis are discussed and suggestions for further research into paediatric schistosomiasis are presented. I will conclude the present chapter with key conclusions for the diagnosis, morbidity assessment and treatment of paediatric schistosomiasis that may aid to the design and monitoring of schistosome control programmes targeting preschool-aged children.

7.2 Infection diagnostics: implications for control

In accordance with the WHO guidelines, quantification of schistosome infection is a pre-requisite for developing intervention protocols for schistosomiasis control programmes (WHO 2002). Accurate detection of schistosome infections is important to ensure that adequate control interventions are implemented and to facilitate timely treatment of infected individuals and hence prevent the development of long-term morbidity sequelae associated with chronic infection (King 2007; Polman 2000; WHO 2007). Childhood infections, if left untreated can have long-term consequences on health in adulthood that may not be reversible by chemotherapy (Stothard *et al.* 2013). The commonly used schistosome infection diagnostic tool, microscopic enumeration of parasite eggs excreted in urine performs poorly in individuals with low infections due to poor sensitivity and cannot detect pre-patent or single sex infections where there is no egg production (Bergquist *et al.* 2009; Engels *et al.* 1996; Mutapi 2011; Smith & Christie 1986; Turner *et al.* 2004), also reviewed elsewhere (Doenhoff *et al.* 2004). In view of the above limitations of the parasitological technique that can result in underestimation of the true prevalence of infection, there is an urgent need for improved additional diagnostic methods, such as described in this

thesis, particularly in young children (typically ≤5 years old) who harbour light infections. In chapter 3 of this thesis, I focused on validating the performance of additional tools for the diagnosis of urogenital schistosomiasis (serological and dipstick detection of microhaematuria) in preschool *vs.* primary school-aged children compared with the 'standard' parasitological examination and their implications for control programmes were also investigated.

It has already been demonstrated in recent epidemiological studies that young children in endemic areas do experience significant exposure to infective waters (Ekpo *et al.* 2012*b*; Mutapi *et al.* 2011; Poole *et al.* 2014; Stothard *et al.* 2011). These findings were confirmed by the results of the present thesis, as shown by a high proportion of children testing positive for detectable anti-cercarial IgM antibodies (see Table 3.2, chapter 3), reaffirming the need for interventions targeting preschool-aged children. Furthermore, I have also confirmed that preschool children do carry significant schistosome infections and that most of these infections are missed by the current standard parasitological diagnostic technique compared to the serological detection method (see Figure 3.3). More importantly, I noted that the discrepancy between infection levels obtained using the two methods also increases with age. By using the serological technique in both preschool and primary school-aged children, I was able to demonstrate that use of parasitology alone might misclassify communities for the praziquantel treatment regimens recommended by the WHO (guided by community infection levels), resulting in fewer treatments than those required (WHO 2002). The consequence of this is that the intervention programmes may fail to achieve maximum effectiveness in terms of prevention and control of schistosomiasis and hence also hamper the vision set out in 2012 by the World Health Assembly resolution 65.21, advocating for the elimination of schistosome transmission (WHO 2010, 2013). In addition, urine dipsticks detected a higher prevalence of infection based on microhaematuria in both age groups compared to the parasitological method (see section 3.8, chapter 3). These findings concur with those of a recent meta-analysis study by King & Bertsch (2013), suggesting that dipstick microhaematuria can be used in conjunction with egg counts to improve detection of schistosome infections in children carrying light infections.

The parasitological technique performed poorly in detecting infection in children with light infections as was shown by the estimate of sensitivity of the test. In contrast, the serological and dipsticks methods performed similarly

in children carrying light or heavy infections and showed good sensitivities in diagnosing infection in both preschool and primary school-aged children compared to the parasitological technique. In addition, it was reassuring to note that the estimated levels of sensitivities of the additional diagnostic tools in the present study were within the ranges reported in several other epidemiological studies of human schistosomiasis conducted in endemically exposed populations (Adesola *et al.* 2012; Bogoch *et al.* 2012; Dawson *et al.* 2013; Kahama *et al.* 1998; King & Bertsch 2013; Kinkel *et al.* 2012). Unsurprisingly, the parasitological diagnostic technique showed a higher specificity compared to serology or dipstick tests as has been reported in other previous studies. The heterogeneity in immune responses between individuals may contribute to false-positive results of the serological test, particularly in older children with long-standing history of infection (van Lieshout *et al.* 2000), resulting in reduced specificity of this technique. Dipsticks are known to yield a high rate of 'false positive' results in children (Lunn & Forbes 2012; Meyers 2004; Patel 2006; Simerville *et al.* 2005), also reported in studies in schistosomiasis (Eltoum *et al.* 1992; King & Bertsch 2013) and this could have contributed to the reduced specificity observed in the present study.

The loss of sensitivity of the parasitological technique in detecting light infections underscores the necessity to refine the methods for diagnosing infection to enable correct epidemiological mapping of prevalence in targeted populations including preschool-aged children. In this thesis, I was able to demonstrate the importance of using sensitive diagnostic methods in children (based on a combination of parasitological and serological methods) to improve diagnostic accuracy, as this has implications on the required treatment regimens for the community recommended by the WHO (WHO 2002). Accurate diagnosis of infection provides a foundation for treatment and management of schistosomiasis (De Vlas *et al.* 2004). Thus, throughout this thesis, I have presented data pertaining to baseline (pre-treatment) infection status determined by both parasitological and serological methods to improve diagnostic accuracy. Since chemotherapy alters the parasite-specific immune responses (Mutapi *et al.* 1998; Rujeni *et al.* 2013), thus, the serological technique is only reliably applicable before treatment.

7.3 Assessment of morbidity in preschool children

The development of morbidity due to schistosomiasis early in childhood may contribute to long-term irreversible consequences if treatment is delayed for too long (Betson *et al.* 2010; Ekpo *et al.* 2012*b*; Stothard *et al.* 2013). Thus, the basis of schistosome control in children is preventative chemotherapy to combat the development of severe morbidity and promote child health and improve cognitive potential of infected children (WHO 2010). However, uncertainties in the levels of morbidity in preschool-aged children is among the reasons why these children have been previously neglected in control programs (reviewed by Mutapi (2015)). In addition, the performance of currently available point-of-care morbidity diagnostics has not yet been evaluated in preschool-aged children. Following the recent recommendation by the WHO for inclusion of preschool-aged children in mass drug administration programmes (WHO 2011*b*), more studies are needed to evaluate tools for assessing morbidity in this age group, which is crucial for the development of effective and sustainable control strategies (Vennervald & Dunne 2006). Having established better a diagnostic approach of schistosome infection in young children, I sought to validate (in chapter 4) the currently available methods for diagnosing schistosome morbidity in large scale population-based studies as recommended by the WHO. These were questionnaire-based reporting of haematuria and dysuria, clinical examination by qualified clinical practitioners, visual inspection of urine for gross/visible haematuria, urinalysis by dipsticks, urinalysis of the albumin-to-creatinine ratio (UACR). I compared the utility of the available morbidity diagnostic tools in preschool *vs.* primary school-aged children and identified markers which can be used in the field to quantify morbidity attributable to *S. haematobium* infection. To my knowledge, these methods have never been directly compared in a single population of children or thoroughly validated in preschool-aged children. In addition, since the study area had no previous history of treatment interventions, the approach presented in this thesis would give a good estimate of the morbidity burden due to schistosomiasis in children.

The results of my study indicated presence of quantifiable levels of morbidity attributable to schistosomiasis in both preschool and primary school-aged children, as determined by the different diagnostic tools. These results are consistent

with another study in Mali, which also reported prevalence of urinary tract pathology in school-aged children due to *S. haematobium* infection (Sacko *et al.* 2011). Such findings reiterate the importance of early anti-schistosomal treatment for endemically exposed children to alleviate development of severe morbidity sequelae. Interestingly, the results demonstrated that the performance of different morbidity detection methods investigated in this thesis was similar in preschool and primary school-aged children infected with *S. haematobium*. The current findings suggest that the available methods could serve as screening tools for schistosome-related morbidity in children aged ≤5 years old, important for the planning of future public health interventions targeting this age group.

A complete dipstick urinalysis involves measurement of several physical and chemical urine attributes that can be indicative of the presence of infection or underlying medical conditions such as urinary tract morbidity, glomerular and renal complications (Simerville *et al.* 2005). Urinary dipsticks have been used extensively in *S. haematobium* endemic areas and have been recommended in several studies as a relatively inexpensive method for detection of infection or related morbidity in large-scale studies (King & Bertsch 2013; Rollinson *et al.* 2005). In this thesis, instead of focusing on individual specific urine attributes as markers of schistosome-related morbidity (a common approach with many studies), I initially investigated several dipstick indicators, namely; nitrites, leukocytes, microhaematuria, proteinuria, and the physical attributes pH and specific gravity, suggested to be important clinical markers of some bacterial/parasitic infections (Carlin 2014). Of the urinary dipstick attributes tested, proteinuria and microhaematuria were the ones found to be related to *S. haematobium* infection. These results are unsurprising; microhaematuria is a well characterized classical sign of urogenital schistosomiasis in children resulting from tissue damage caused by exiting parasite eggs (Adesola *et al.* 2012; Gryseels *et al.* 2006). Renal changes leading to nephrotic syndrome have been reported in individuals chronically infected with *S. haematobium* and this is commonly clinically manifested by presence of proteinuria (Bichler *et al.* 2006; Eknoyan *et al.* 2003; Stete *et al.* 2012).

One approach used to determine morbidity in schistosome-endemic areas is questionnaire evaluation of morbidity, mainly haematuria (presence/absence), recommended by the WHO (WHO 2002). In infected individuals, presence of morbidity can also be perceived through dysuria (painful urination) which can

be assessed by means of questionnaires (Lengeler *et al.* 2002). I compared the levels of haematuria obtained from questionnaire *vs.* those measured by dipstick (microhaematuria) or visual urine examination (visible haematuria) and the results showed that the dipsticks detected more haematuric cases than the questionnaires, which in turn detected more cases than the visual urine examination (see Figure 4.3). These results match those observed in an earlier meta-study by van der Werf & de Vlas (2004) conducted in older children and adult populations. The high prevalence of dipstick detected microhaematuria may be explained by the high sensitivity of the dipsticks as already observed in chapter 3 and reported in other studies (see comprehensive review by King & Bertsch (2013)). Furthermore, the questionnaires showed some bias in reporting between preschool children where the answers were provided by the parents/guardians and in primary school children who responded to the questions themselves. This could not be adjusted for in the statistical analysis, hence caution must be exercised when extrapolating these results.

Since the different markers are general indicators of morbidity that can arise from different disease conditions, in order to investigate the potential utility of the five diagnostic methods in assessing schistosome-related morbidity in this thesis, I employed the method of attributable fractions. This approach is preferable to results based only on the apparent morbidity prevalence, in that the attributable fraction estimates are adjusted for background causes of the non-specific symptoms and hence more meaningful for biological interpretations (Guyatt *et al.* 1995). Based on these results, I was able to identify UACR as the most reliable tool for detecting schistosome-related morbidity, followed by dipsticks, visual urine inspection, questionnaires and lastly clinical examination. More importantly, the potential of UACR as a morbidity assessment tool in children was further demonstrated when the analysis was stratified by age group (i.e., 1–5 years and 6–10 years old). However, it will be of practical importance in future studies with large enough sample sizes, hence high enough power, to confirm the results of stratified analysis for generalizability of the present findings. The strong association between albuminuria (detected by UACR) and dipstick-detected microhaematuria and proteinuria further suggests that these markers can be used in combination to improve the diagnosis of morbidity in children infected with *S. haematobium* in the field. These findings are of public health importance as these tools can be used to screen affected children for treatment and thereby

facilitating timely interventions. These tools can also be used to assess the impact of treatment on morbidity (chapter 5).

7.4 Optimal treatment regimen: implications for control

The antihelminthic drug praziquantel (PZQ) is effective against all schistosome species and it presently forms the backbone for all public health interventions aimed at prevention and control of schistosomiasis in endemic areas (Stothard *et al.* 2014; WHO 2013). The WHO recommends different treatment regimens (e.g. annually, biennially) depending on the schistosome infection intensity levels in the community, usually assessed by surveying primary school children (WHO 2002). However, there is currently no evidence base to inform the PZQ regimen when extended to include preschool children. Having established better diagnostic approaches of schistosome infection and morbidity (chapters 3 and 4), I assessed the short term effects of treatment on infection and morbidity markers (microhaematuria, proteinuria and albuminuria) by following treated children 12 weeks post-treatment. The immediate health benefits of PZQ treatment in children aged 6–10 years of age have already been documented (Koukounari *et al.* 2007; Midzi *et al.* 2008; Sacko *et al.* 2009) and therefore by including 6–10 year olds in the present study, I was able to determine whether the effects of PZQ treatment on health and morbidity measures were age dependent. Furthermore, I sought to investigate ways of refining the existing drug regimen to improve the current and future health of preschool children by comparing the impact of single biennial *vs.* annual treatment with PZQ over a period of 24 months on the selected markers of schistosome-related morbidity.

The results of chapter 5 showed that a single treatment with the recommended dose of PZQ (40 mg/kg body weight) not only reduced infection levels, but was also efficacious at significantly reducing morbidity markers in both preschool and primary school-aged children within 12 weeks and 12 months of treatment. However, some post-antihelminthic treatment changes in morbidity markers may take time to appear, possibly due to slow bladder and urinary tract tissue repair (Stete *et al.* 2012). In the present study, microhaematuria levels showed a significant decline only after 12 months compared to other morbidity

markers which showed a significant change within 12 weeks of treatment. Similar observations of persistent haematuria for periods of up to 6 months following curative chemotherapy have been reported in other studies (Nkulila *et al.* 1999). Interestingly, I did not observe significant differences in the effects of treatment in reducing morbidity markers between preschool and primary school-aged children. The current findings therefore further support the idea that PZQ offers a realistic opportunity as a control strategy for schistosome infections in preschool children and reducing the current and future burden of morbidity related to schistosomiasis (Koukounari *et al.* 2007; Mutapi *et al.* 2011; Stothard *et al.* 2013). In agreement with observations from previous studies (King 2006), I was able to demonstrate the sustained benefits (over a period of 2 years) of PZQ treatment in reversing morbidity in children. Such findings reaffirm the need for preventive chemotherapy in preschool-aged children through ongoing MDAs for effective and sustainable control as recently recommended (WHO 2011*b*). Nevertheless, it is important that treatment administration is supported by other complementary control measures such as improved sanitation to reduce the burden of schistosomiasis in endemic regions (Fenwick *et al.* 2009; Ross *et al.* 2014).

In order to determine the optimal treatment regimen required to significantly reduce morbidity attributable to schistosomiasis, I compared the changes in schistosome-related morbidity markers in children who received biennial *vs.* annual PZQ treatment over the 24 months study period (see section 5.4.2, chapter 5). The current study did not demonstrate differences in the efficacy of biennial *vs.* annual PZQ treatment in reducing schistosome-related morbidity across the two age groups. These findings suggest that the biennial praziquantel treatment regimen currently recommended by the WHO for primary school-aged children in medium schistosome infection areas is sufficient for preschool-aged children. The current findings support those of previous *S. haematobium* studies in Ghana and Burkina Faso among primary school and older children (6–15 years), indicating that a single dose of praziquantel has lasting effects on schistosome-related morbidity (Koukounari *et al.* 2007; Mott *et al.* 1985). My present findings provide new information that would be important in the planning and implementation of optimal control strategies targeting preschool children and help to inform resource allocation in ongoing programmes to achieve the goals of sustainable control of schistosomiasis (WHO 2013).

7.5 Serological biomarkers of morbidity

The recent recommendation for the inclusion of preschool children in control programmes (WHO 2011*a*) has heightened the need for tools that can assist in detecting early morbidity due to schistosomiasis. Throughout this thesis, I have attempted to identify and validate several methods to improve the diagnosis of infection and related morbidity in young children. Early detection of circulating biomarkers associated with initial immune-pathogenesis in children could help in identifying and preventing disease progression in the later stages of life (King 2007). In addition, such biomarkers could enhance understanding of the mechanisms of development of morbidity, which can be important for the implementation of timely and effective interventions (Vennervald & Dunne 2006). Thus, in chapter 6, I simultaneously examined several biomarkers known to play an active role in different phases of the inflammatory process in a population of young children endemically exposed to *S. haematobium* infection. The relationships between CHI3L1, CRP, ferritin, resistin and levels of infection (as diagnosed using the methods described in chapter 4), inflammatory cytokine responses (IL-4, IL-5, IL-10, IL-13 and IFN-γ), and point-of-care markers of morbidity (microhaematuria, proteinuria and albuminuria) already identified and validated in chapters 4 and 5 were investigated to determine the value of these inflammatory biomarkers as markers for the assessment of schistosome-related pathology.

The inflammatory environment during infection is characterized by a range of different responses rather than a single immune response marker (Bourke *et al.* 2012). In my current study, the established positive association between the different biomarkers of inflammation and their correlation with systemic levels of pro-inflammatory cytokine responses suggested an ongoing inflammatory process in children naturally exposed to schistosome infections. In particular, CHI3L1 and CRP, together with systemic cytokines IL-13 (pro-inflammatory) and IL-10 (playing a regulatory role) showed the strongest contribution to the inflammatory environment in this population. However, I did not find a significant relationship between inflammatory biomarkers and current point-of-care markers of morbidity attributable to schistosomiasis. There are several possible explanations for this result as noted in chapter 6, some of which may warrant further investigation in future studies involving both preschool children as well as adult populations. The

lack of association could be that the low schistosome infections in children are associated with lower inflammatory responses not detectable by these markers, raising the possibility that the levels of circulating inflammatory biomarkers in my population were too low to have major physiological or biochemical consequences such as proteinuria and nutritional deficiency observed in other studies (Appleby *et al.* 2012; Ayoya *et al.* 2010; Friis *et al.* 1996; Reilly *et al.* 2012). Given that my study population consisted of the youngest members of the community (≤10 years) with a shorter cumulative history of exposure to schistosome infection relative to older members of the population, it could be that the majority of these inflammatory biomarkers may be closely associated with the severe pathology (Zakynthinos & Pappa 2009) that may not yet be observed in most of these children in the current population. The non-specific nature of the inflammatory biomarkers also allows the possibility that other potentially confounding bystander antigens such as *Plasmodium* reported in Zimbabwean populations (Rujeni *et al.* 2013) were causing their elevation in this population.

Assessment of the effect of treatment with praziquantel was limited to the two biomarkers (CHI3L1 and CRP) identified as the most dominant players in the inflammatory processes observed in this study and hence indicators of the risk of schistosome-related morbidity (see section 6.7). PZQ treatment in infected children did not result in significant changes from baseline in serum CRP levels 12 months after chemotherapy, but resulted in a significant elevation of CHI3L1 levels, in contrast with observations from recently published studies in schistosomiasis (Appleby *et al.* 2012; Ayoya *et al.* 2010). As I have already highlighted above, the application of these circulating protein biomarkers as tools for assessing the impact of interventions is limited by the wide variety of agents causing their elevation in serum and the variations in individual host immune responses (Mutapi 2001; Zakynthinos & Pappa 2009). Further research is warranted to better define the effects of PZQ in levels of protein biomarkers of inflammation in young children by examining changes in their serum concentration over shorter times before and after treatment compared to changes in older individuals. Nonetheless, by examining several serological markers simultaneously in a single population, my findings add to the current limited understanding of the relationship between circulating biomarkers of inflammation and pathogenesis of schistosomiasis.

7.6 Issues of methodological approaches

My studies were carefully designed and data collected as planned in the study protocol without deviations to guard against drawing faulty conclusions. Exposure to the parasite in the current study was measured using IgM antibody responses directed against the larval stages of the parasite (cercariae). However, cross-reactivity with other parasitic antigens cannot be completely ruled out (Ndhlovu *et al.* 1996). In my study I could have further explored details of exposure to infective water to improve estimation of exposure rates among these preschool children, captured using approaches such as direct observation and global position system (GPS) logging of actual water contact behaviour that have been successfully used in other studies of schistosomiasis (Brooker *et al.* 2009; Chandiwana & Woolhouse 1991). Including vital statistics of health measures such as temperature, blood pressure, heart rate and respiratory rate as well as anthropometric data (weight, height, arm circumference and head circumference) of each child measured before treatment and at 12 and 24 months after each treatment would have enabled me to assess the impact of schistosomiasis on childhood growth and physical activity. In addition to the serological biomarkers of inflammation, the WHO recommends inclusion of indicators of iron deficiency and anaemia contributing to malnourishment in children (WHO 2005). Measurement of markers such as haemoglobin, serum transferrin receptor (sTfR), the hormone hepcidin, which regulates iron homeostasis (Lee & Beutler 2009), and eosinophils cationic protein (ECP) may aid in unravelling the immunopathology of schistosomiasis in preschool and primary school-aged children.

The limitations of the different statistical methodologies performed to investigate associations between several inflammatory biomarkers and immune correlates of pathology in my study are mainly to do with the issues of multicollinearity and multiplicity. A more powerful and flexible alternative technique would be the structural equation modelling (SEM) technique (Pugesek *et al.* 2003), as it allows multiple measures to be associated with a single latent construct. SEM is largely confirmatory, rather than exploratory (Pugesek *et al.* 2003), thus it will be interesting to confirm whether the established relations between serological markers of inflammation, cytokine responses and point-of-care markers of schistosome-related morbidity are valid using this technique.

7.7 Future prospects and recommendations

In this thesis I have demonstrated that the shortcomings of the parasitological method result in the underestimation of the levels of infection in preschool children, and that diagnosis can be improved by use of the more sensitive serological method in addition to the standard parasitological diagnostic technique. Despite the limitations described here, egg counts continue to be utilized as a proxy for the worm burden. However, in low infection settings this may not be a good reflection of the true levels of the worm load. Previous studies in older children and adults have suggested a strong relationship of urine and serum circulating antigens with worm burden (van Lieshout *et al.* 2000; Van Lieshout *et al.* 1995). Further research into other direct measures such as circulating anodic antigen (CAA) and circulating cathodic antigen (CCA) in serum and urine as potential indicators of the true worm load will further enhance the knowledge of the burden of schistosomiasis in young children aged 5 years and below.

I have demonstrated that PZQ is efficacious in preschool-aged children. However, it still remains to be established whether antihelminthic treatment of preschool children induces immune responses associated with protection against re-infection as has been reported in adult populations (Mutapi *et al.* 2005). Thus, I propose further studies aimed at investigating a treatment strategy that will induce protective immune responses against schistosome re-infection in children aged 5 years and below. With improved knowledge of efficacy of treatment, the ultimate goal of elimination of morbidity burden associated with schistosomiasis in children is achievable.

The involvement of serum biomarkers and other immune correlates in the biological processes of inflammation observed in this thesis suggests that CHI3L1 and CRP can be potential early predictors of schistosome-related pathology. I recommend further research efforts with larger cohorts of children chronically exposed to schistosome infection to establish the relationship between these biomarkers and presence of schistosome-related morbidity by following changes in the serum levels of these biomarkers over shorter time periods. Lastly, in terms of control policy and practice, based on my current findings demonstrating the benefits of PZQ in children, I encourage up-scaling of efforts (see section 7.8) towards refining the timing of the treatment to optimize the health benefits of

antihelminthic treatment in preschool-aged children using the currently available tools for control.

7.8 Implications for control and policy

Approximately 90% of all malaria incidence and mortality occur in Africa, where one child in 10 dies before the age of 5 years from malaria (WHO 2014). In addition, in these tropical regions there is an overlapping distribution of helminth infections and micronutrient deficiencies, contributing to anaemia among children, a major cause of poor growth and development (WHO 2011*c*). According to the Global Progress Report on Vitamin and Mineral deficiency, more than half of the population in Africa lack critical vitamins and minerals (UNICEF 2010). Vitamin A deficiency is a common form of micronutrient malnutrition affecting preschool-aged children contributing to child mortality in these areas (WHO 2011*c*). Several childhood health promoting activities such as the Expanded Programme on Immunisation (EPI), distribution of vitamin and mineral supplements, administration of STH antihelminthics (albendazole and mebendazole) have been put in place in several African countries, including Zimbabwe (Gadaga *et al.* 2009; UNICEF 2010). In light of my present findings, these ongoing programmes could expand to include praziquantel treatment for children under 5 years of age in an effort to address multiple diseases at once (Hotez *et al.* 2006; WHO 2013).

Over the past three decades, praziquantel, which is currently the only recommended drug for infection and disease has been used successfully to control schistosome infection and disease in endemic countries (WHO 2002). The recent scaled-up efforts for the control of neglected tropical diseases by various stakeholders and commitment from the pharmaceutical industry to donate PZQ tablets yearly to school-aged children (see (Mutapi 2015)) has led to a significant increase in the accessibility of PZQ as control tool in large-scale mass drug administration (MDA) programmes (WHO 2013). As highlighted in a recent review by Mutapi (2015), the inclusion of preschool-aged children in currently ongoing schistosome programmes is slow. One reason for the slow uptake is concerns about the large size of PZQ tablets, which make it difficult for young children to swallow them (WHO 2006). In addition, the dextro isomer which is part of the PZQ compound (it consists of equal parts of levo and dextro-isomers) gives the tablet a bitter taste that may cause gagging or vomiting (Meyer *et al.*

2009). As of present, inventions to improve and deliver oral formulations of PZQ for paediatric interventions are currently underway through partnership of Merck KGaA, Astellas Pharma, and the Swiss Tropical and Public Health Institute. These concerted efforts are directed at overcoming the operational hurdles slowing the uptake of preschool children in MDA programmes (Mutapi 2015; Tuhebwe *et al.* 2015).

Lastly, it is worth noting that part of my research work presented in this thesis is already influencing research and policy development for control. Results of my fieldwork informed the formulation of the Zimbabwe's national schistosome and soil transmitted helminths control program drafted in 2012 (Chimbari 2012), that facilitated treatment of preschool-aged children within the first year of the ongoing MDA in the country and making it one of the first national control programmes to include this neglected age group.

7.9 Final conclusions

My thesis has generated several results of research interest, of importance for practitioners, policy makers and stakeholders involved in the control of schistosomiasis. These findings are timely because of the current global drive to address the health iniquity created by the paucity of information on the disease in preschool children (aged 1–5 years old):

- The study has confirmed that preschool children carry significant schistosome infections, and that most of these infections are missed by the current standard egg count diagnostic method. By using the more sensitive SEA IgM serological diagnostic method, I have been able to demonstrate that parasitology might misclassify communities for the treatment regimens recommended by WHO (guided by community infection levels) resulting in fewer treatments than those required. These findings further reiterate the urgent need for schistosome infection control in this age group and demonstrate the implications of poor diagnosis of infection for control strategies adopted.

- Preschool-aged children can be effectively screened for schistosome-related morbidity using the same diagnostic tools applicable in school-aged children and adult populations. From my studies, UACR is recommended as the

best choice for rapid assessment of morbidity attributable to schistosomiasis in children under field conditions. Clinical examination is too costly and requires advanced skills, but is poor at detecting morbidity attributable to schistosome infections, hence not ideal for large-scale field use.

- A single treatment with a standard dose of praziquantel (40 mg/kg) is efficacious at significantly reducing infection and morbidity in children. More importantly, the biennial treatment regimen currently recommended by the World Health Organization for primary school-aged children in the medium schistosome infection areas is sufficient and applicable to preschool-aged children.

- The involvement of serum circulating protein biomarkers in the biological processes of inflammation in naturally exposed children suggests that these markers can be potential early predictors of the risk of schistosome-related pathology. In this thesis, CRP and CHI3L1 were identified as the potentially useful inflammatory biomarkers for the assessment the risk of morbidity due to schistosomiasis.

In summary, the findings of this thesis highlight the need for the refinement of existing diagnostic methods for accurate detection of infection and morbidity in children. This will enable appropriate and timely treatment strategies, aimed at improving the current and future health of preschool aged-children to be implemented. The findings presented here will aid researchers and other stakeholders in making informed choices about intervention tools for control programmes targeting young children.

Appendices

Appendix A

Antibody assays protocol

Serum levels of IgM antibodies targeted against CAP and SEA were measured using indirect ELISA method, all of which have the same principle protocol as described below:

1. **Plate antigen coating:** Assay plates (96-well microtiter, Greiner Bio-One, UK) were coated with 100 μL/well of antigen at 5 μg/mL (for CAP), and 10 μg/mL (for SEA) diluted in carbonate bicarbonate buffer at pH 9.6, covered with cling film and incubated overnight at 4°C.

2. **Blocking:** Plates were washed once in phosphate buffered saline (PBS)/0.03% Tween 20, which was used for all washes, emptied and blocked with 200 μL/well of skimmed milk for an hour at 37°C to avoid non-specific binding.

3. **Serum sample:** The plates were washed three times in wash buffer to remove unbound blocking agent, and 100 μL/well of sera were added in duplicate using sample dilutions 1:100. The plates were incubated at 37°C for two hours. Secondary antibodies were diluted in blocking buffer to minimize the non-specific binding effect that could occur on the plate during the assay.

4. **Detection antibody:** The plates were washed again three times in wash buffer to remove excess serum components, and 100 μL/well of secondary antibody (horseradish peroxidase (HRP) conjugated) was added at a dilution of 1:1000. The plates were incubated at 37°C for 1 hour.

5. **Substrate solution:** Plates were washed six times and 100 μL/well of 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) peroxidase

substrate solution were added. The plates were incubated at 37°C covered with aluminium foil and an enzyme-substrate reaction time of 15 minutes was allowed to take place before plates were read.

6. **Reaction stop and detection:** The colorimetric reactions were stopped by adding 25 μL x well of stop solution (25% hydrogen chloride, HCl). The results were expressed as the mean optical density (OD) value of the duplicate assays quantified by an Emax microplate reader (Molecular Devices, USA) at a wavelength of 405 nm.

All the chemicals used for the assays were supplied by Melford (Melford Laboratories Ltd., UK) or Sigma-Aldrich (Sigma-Aldrich Corporation, UK) unless otherwise stated.

Appendix B

Dipstick results coding in the field

Table B.1: Calibration of urine attributes measured using Uristix® reagent strips following manufacturer's guidelines and re-coded for easy referencing and data quality management in the field.

		Urinary Dipsticks Data coding in the field						
Marker	**Unit**	**1**	**2**	**3**	**4**	**5**	**6**	**7**
Urobilinogen	mmol/L	0.1	1	-	2	4	8	–
Glucose	mmol/L	neg	±100	++500	+++1000		–	–
Bilirubin		neg	–	+	++	+++	–	–
Ketones	mmol/L	neg	±5	+15	++40	+++100	–	–
Specific Gravity		1.1000	1.0050	1.0100	1.0150	1.0200	1.0250	1.0300
Blood	RBC/μL	neg	+10 (hemolysis)	++50	+++250	+10 (non-hemolysis)	++50	–
pH		5.0	6.0	6.5	7.0	8.0	9.0	–
Protein	mg/dL	neg	trace	+ 30	++ 100	+++300	++++1000	–
Nitrite		neg	trace	pos	–	–	–	–
Leukocytes	WBC/μL	neg	+25	++75	+++500	–	–	–

Appendix C

Evaluating cut-offs for serological diagnosis

C.1 Antibody cut-off levels

Potential bias can arise as a result of subjective cut-off thresholds or lack of robustness in the chosen cut-offs to slight changes in sampling variability leading to over- or underestimation of important epidemiological parameters such as infection prevalence (Greiner *et al.* 1994). Since the choice of these cut-offs can influence the inferences derived from subsequent analyses, I evaluated the utility of the cut-off values determined from a pool of serum specimens obtained from healthy donors (Figure C.1). The arithmetic mean is sensitive to outlying observations, thus if the samples used to determine cut-offs are over-dispersed, the computed thresholds may become too high, consequently failing to correctly classify seropositive individuals. I investigated the influence of each observation on the overall mean of the negative controls using the jackknife procedure (Sprent & Smeeton 2001). The jackknife procedure is a cross-validation technique to estimate the bias of an estimator (e.g., the mean OD value), through a series of iteration steps ("omitted" or "leave one out"). Firstly, the mean is estimated from the whole sample, and then one observation is dropped from the sample, and the mean is estimated from the reduced sample. The jackknife bias of the mean can then be determined by assessing the variability of the resulting sample of estimates with one observation omitted (Sprent & Smeeton 2001). The robustness of these cut-offs to changes in sample variability was assessed by shifting each cut-off values

by a margin of 5% (i.e. change, δ=-5%; +5%; +10%), and assessing the impact of the change on the fraction seropositive.

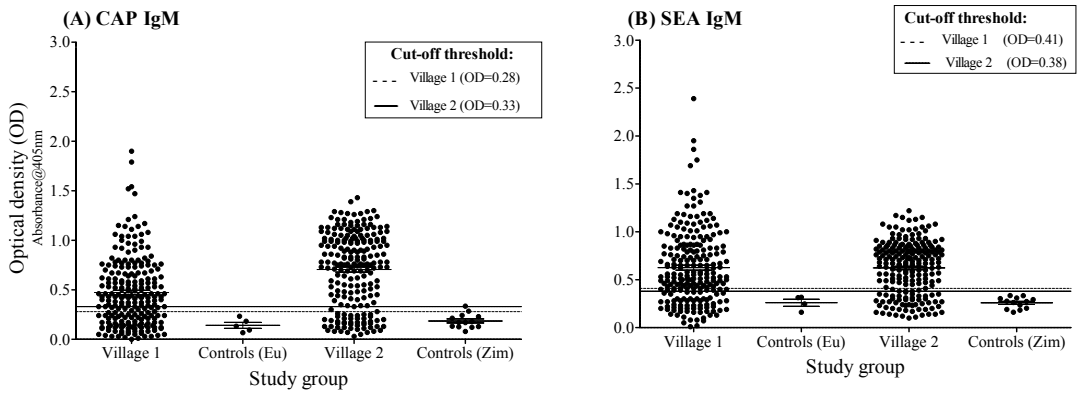


Figure C.1: Distribution of IgM antibody levels directed against schistosome cercariae and egg antigens for the study population by village of residency and negative controls. The dashed line (- - -) represents the cut-off threshold for children from village 1 derived from the schistosome negative European (Eu) controls, and the solid line (—) represents the cut-off threshold used in the diagnosis of children from village 2 derived from healthy Zimbabwean (Zim) donors.

C.2 Identifying influential observations

Since the ELISAs for serum samples of children from the two study villages were conducted at different time periods, thus to avoid bias due to experimental variability, the cut-offs were investigated by village of permanent residency. None of the individual IgM antibody titres from the negative controls were above the cut-off thresholds for both CAP and SEA (Figure C.1). The variability in the estimated mean of the negative controls using the jackknife (i.e. with one observation omitted) did not change markedly as shown by the root-mean-square errors (RMSE) in Figure C.2, indicating no potential bias in the estimated mean OD due to individual observations for IgM CAP or SEA control samples.

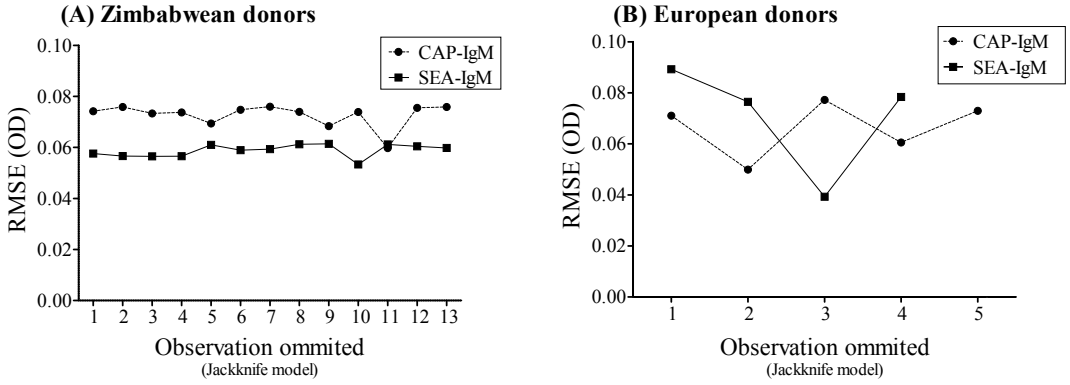


Figure C.2: Change in root-mean-square-error (RMSE). Investigation with one observation omitted in the Jackknife procedure to assess the bias of the mean optical density (OD) used to compute the cut-offs.

C.3 Fraction positive

Varying the cut-offs did not result in a change in the proportion of seropositive children (Figure C.3). In addition, reducing the cut-off levels by 5% resulted in only 2 CAP false positives and no SEA false positives, as determined from the negative controls. These findings indicated that the chosen cut-offs were robust to reasonable shifts in sample variability.

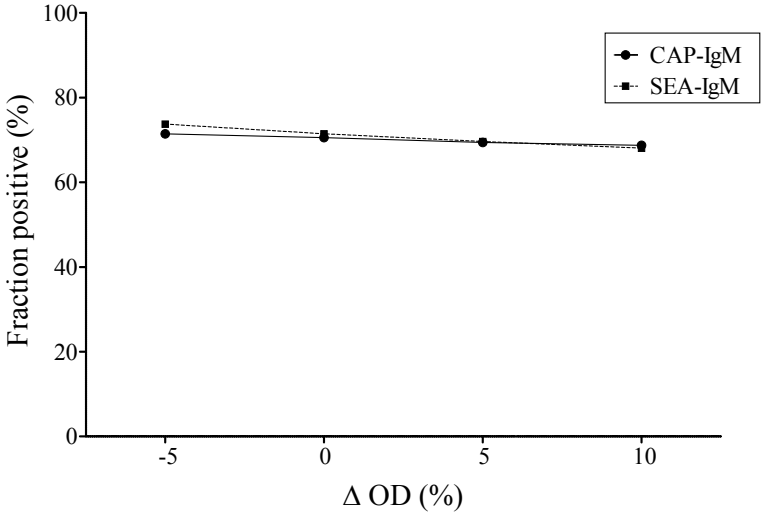


Figure C.3: Change in the fraction positive children for CAP and SEA IgM with cut-off values scaled upwards or downwards by margins of 5% (Δ OD).

Appendix D

Bayesian methods

D.1 Estimation of diagnostic test parameters

In the absence of a gold standard test, the true disease status (D) of the study subjects is unknown and this can bias the estimates of sensitivity and specificity as a result of misclassification (Hui & Zhou 1998). The model presented here considers a two tests, one population scenario (see Dendukuri & Joseph (2001), Branscum *et al.* (2005) for detailed descriptions). Under this setting, two diagnostic tests are each applied to the same subject, assuming the the two test outcomes for each subject are conditionally independent on the infection status. The resulting test outcomes can be cross-classified in a single 2×2 contingency table as shown in Table D.1.

Table D.1: Representation of two infection diagnostic test outcome combinations. Cross-classification according to the infection status of each detected by test 1 (T_1) and test 2 (T_2), neither of which is a perfect gold standard.

		Diagnostic Test 2		
		$T_2 = 1$	$T_2 = 0$	**Total**
Diagnostic Test 1	$T_1 = 1$	n_{11}	n_{10}	$n_{11} + n_{10}$
	$T_1 = 0$	n_{01}	n_{00}	$n_{01} + n_{00}$
Total		$n_{11} + n_{01}$	$n_{01} + n_{00}$	n

The parameters of interest to compare the performance of the two diagnostic tests include: 1) the population prevalence (π); 2) the sensitivities (Se_1 and Se_2); and 3) the specificities (Sp_1 and Sp_2) of each test. For any individual chosen at

random from the study population, infected or uninfected ($D = 1$ or $D = 0$), each diagnostic test can either be positive or negative. Subsequently, this results in eight possible combination of outcomes, conditional on the unobserved (latent) true infection status, each with an associated probability given by:

$$\begin{cases} P(T_1 = 1, T_2 = 1|D = 1) = Se_1Se_2 \\ P(T_1 = 1, T_2 = 0|D = 1) = Se_1(1 - Se_2) \\ P(T_1 = 0, T_2 = 1|D = 1) = (1 - Se_1)Se_2 \\ P(T_1 = 0, T_2 = 0|D = 1) = (1 - Se_1)(1 - Se_2) \\ P(T_1 = 1, T_2 = 1|D = 0) = (1 - Sp_1)(1 - Sp_2) \\ P(T_1 = 1, T_2 = 0|D = 0) = (1 - Sp_1)Sp_2 \\ P(T_1 = 0, T_2 = 1|D = 0) = Sp_1(1 - Sp_2) \\ P(T_1 = 0, T_2 = 0|D = 0) = Sp_1Sp_2 \end{cases} \tag{D.1}$$

Let the latent true number of infected individuals for each of the combination of the two diagnostic test results be denoted by $Y_{t_1t_2}$ (where $t_1, t_2 = 0, 1$), i.e. $[Y_{11}, Y_{10}, Y_{01}, Y_{00}]$. It then follows from equation D.1 and Table D.1 that, the multinomial likelihood function of the observed data given the latent data can be written as:

$$\begin{aligned} L &= P(n_{11}, n_{10}, n_{01}, n_{00}|\pi, Se_1, Se_2, Sp_1, Sp_2, Y_{11}, Y_{10}, Y_{01}, Y_{00}) \\ &\propto [\pi(Se_1Se_2)]^{Y_{11}}[\pi(Se_1(1 - Se_2))]^{Y_{10}} \\ &\times[\pi((1 - Se_1)Se_2)]^{Y_{01}} \\ &\times[\pi((1 - Se_1)(1 - Se_2))]^{Y_{00}} \\ &\times[(1 - \pi)((1 - Sp_1)(1 - Sp_2))]^{n_{11}-Y_{11}} \\ &\times[(1 - \pi)((1 - Sp_1)Sp_2)]^{n_{10}-Y_{10}} \\ &\times[(1 - \pi)(Sp_1(1 - Sp_2))]^{n_{01}-Y_{01}} \\ &\times[(1 - \pi)(Sp_1Sp_2)]^{n_{00}-Y_{00}} \end{aligned} \tag{D.2}$$

To enable comparisons to be made between the diagnostic tests while accounting for uncertainty in the test properties (sensitivity and specificity), the Bayesian modelling approach is invoked (Branscum *et al.* 2005; de Clare Bronsvoort *et al.* 2010; Dendukuri & Joseph 2001; Joseph *et al.* 1995). In the Bayesian framework, prior distributions of all unknown parameters are initially specified. The observed data, through the likelihood function, are then

combined with the prior distributions to derive the posterior distributions. The posterior distributions provide estimates of the test properties, taking into account the information provided by the data (Joseph *et al.* 1995). Thus, improved inferences on all parameters can be drawn simultaneously based on the posterior distribution (Ahmad 2010; Joseph *et al.* 1995).

D.2 Bayesian model diagnostics

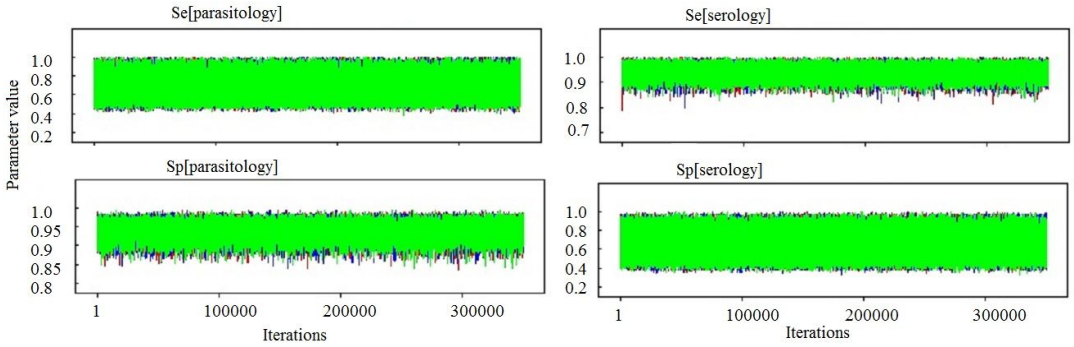


Figure D.1: Trace plots of time series for parameter estimates for parasitological and serological diagnostic techniques to detect *S. haematobium* infection (Se=sensitivity; Se=specificity). The three chains of the MCMC procedure converged to the same solution.

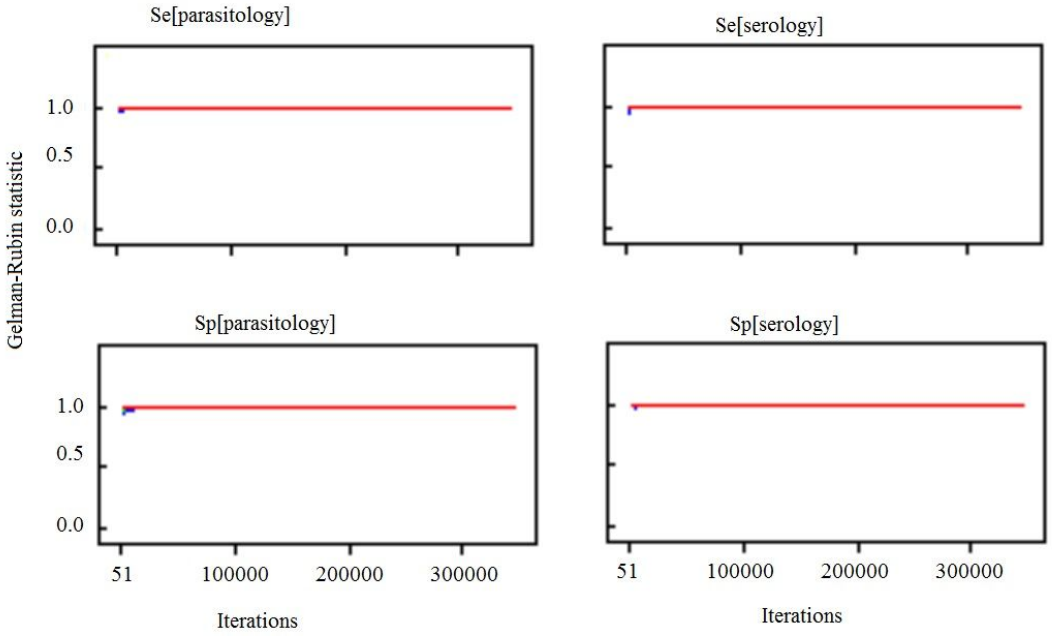


Figure D.2: Gelman-Rubin statistic plots for parameter estimates for parasitological and serological diagnostic techniques to detect *S. haematobium* infection (Se=sensitivity; Se=specificity). The three MCMC chains converge to 1, showing no evidence of non-convergence issues.

Appendix E

SAS software code

E.1 The basic SAS code for the GLMM

```
ods graphics on;
PROC GLIMMIX data=<> oddsratio plots=oddsratio;
CLASS Sex Agegroup Infection Treatment;
MODEL Morbidity=Sex|Age_group|Infection|Treatment / dist=binary;
RANDOM intercept /subject=<Study_ID>;
RUN;
```

Appendix F

Distribution of serological biomarkers of inflammation

F.1 Frequency histograms of serological inflammatory biomarkers and plasma cytokines

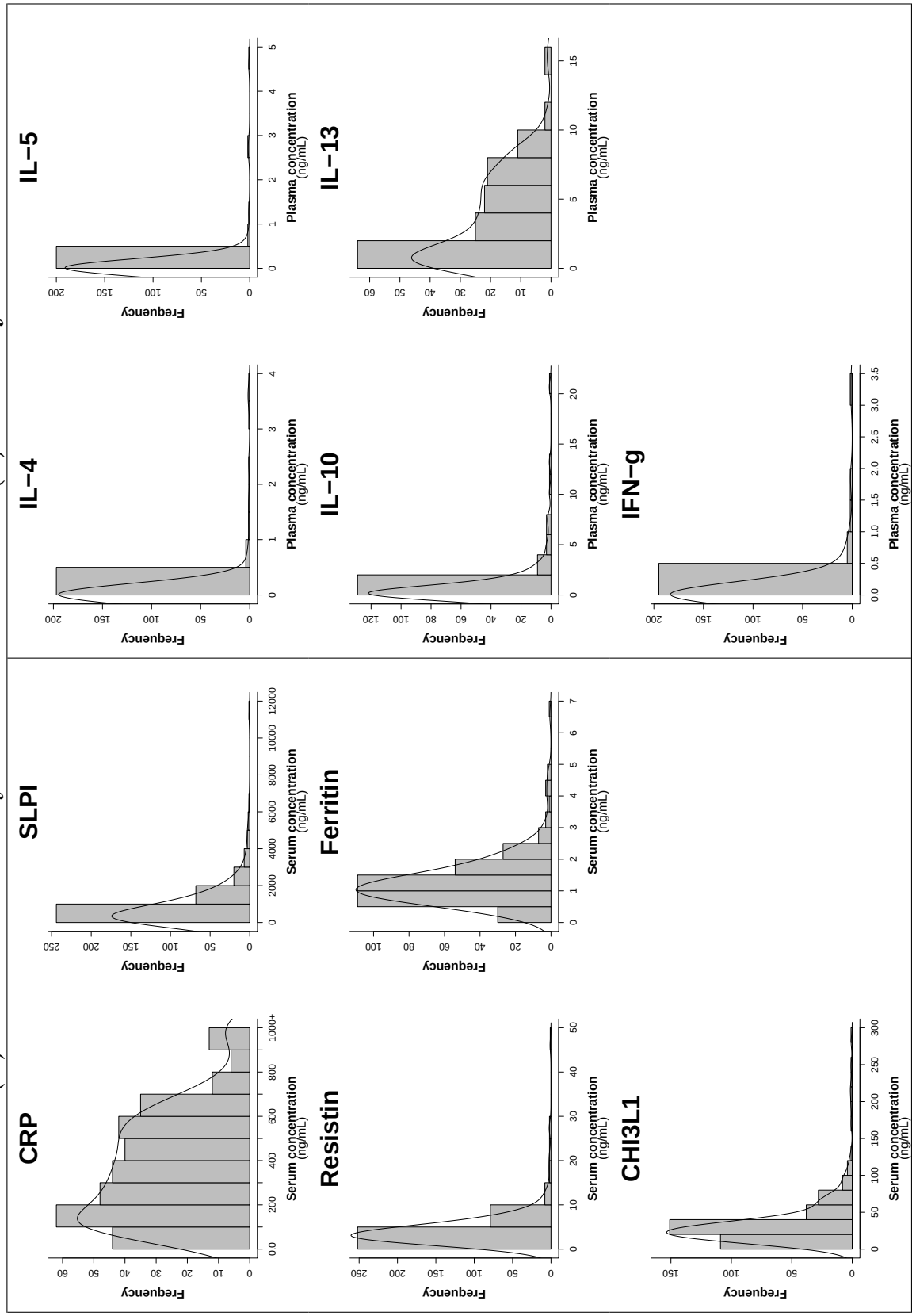


Figure F.1: Frequency histograms of levels of inflammatory biomarkers and plasma cytokines with density curves showing the empirical distributions.

F.2 Box-Cox power transformation

I used the Box-Cox power transformations to derive the potentially optimal transformations of the inflammatory biomarkers. Assuming a simple general linear model with a dependent variable of interest (Y_i):

$$Y_i = \beta_0 + \beta_1 X_i + \varepsilon_i, \tag{F.1}$$

where $\varepsilon_i \sim \mathcal{N}(0, \sigma^2)$. The family of power transformations (λ) as suggested by Box & Cox (1964) is formulated as follows:

$$Y_i^{(\lambda)} = \begin{cases} \frac{(Y_i^{\lambda}-1)}{\lambda} & \text{if } \lambda \neq 0 \\ log(Y_i) & \text{if } \lambda = 0 \end{cases} \tag{F.2}$$

The log-likelihood of model F.2 used to derive the transformations can be written as:

$$log\ L = -\frac{n}{2}\log(2\pi) - n\log\sigma - \frac{1}{2\sigma^2}\sum_{i=1}^{n}\left[Y_i^{(\lambda)} - (\beta_0 + \beta_1 X_i)\right]^2 + (\lambda - 1)\sum_{i=1}^{n} logY_i \tag{F.3}$$

The recommended power transformation λ-values are shown in Table F.1.

Table F.1: Recommended Box-Cox power transformations. Reference source: (Kutner *et al.* 2005).

Family of power	Transformation	λ-value
$Y^{(\lambda)}=Y^2$	Square	$\lambda = 2$
$Y^{(\lambda)}=Y$	None	$\lambda = 1$
$Y^{(\lambda)}=Y^{1/2}$	Square-root	$\lambda = 0.5$
$Y^{(\lambda)}=\mathbf{log(}Y\mathbf{)}$	**Natural log**	$\lambda = 0$
$Y^{(\lambda)}=1/Y^{1/2}$	Inverse square-root	$\lambda = -0.5$
$Y^{(\lambda)}=1/Y$	Reciprocal	$\lambda = -1$
$Y^{(\lambda)}=1/Y^2$	Inverse square	$\lambda = -2$

Table F.2: Calculated Box-Cox power transformation values for inflammatory biomarkers and cytokines.

Response variable	λ-value	Recommended
Inflammatory biomarkers		
CHI3L1	0.14	log-transformation
CRP	0.02	log-transformation
Ferritin	0.22	log-transformation
Resistin	-0.14	log-transformation
SLPI	0.22	log-transformation
Cytokine responses		
IL-4	-0.20	log-transformation
IL-5	0.00	log-transformation
IL-10	0.00	log-transformation
IL-13	0.30	log-transformation
IFN-γ	0.20	log-transformation

Based on the recommended transformations displayed in Table F.1 and for meaningful biological interpretations, the log-transformation was chosen as the best method for these inflammatory biomarkers and cytokines. The log-transformation also enabled me to make comparisons of data on levels of biomarkers presented in this thesis to those reported in other published studies using the similar transformation scale.

F.3 Proportion of detectable levels of biomarkers

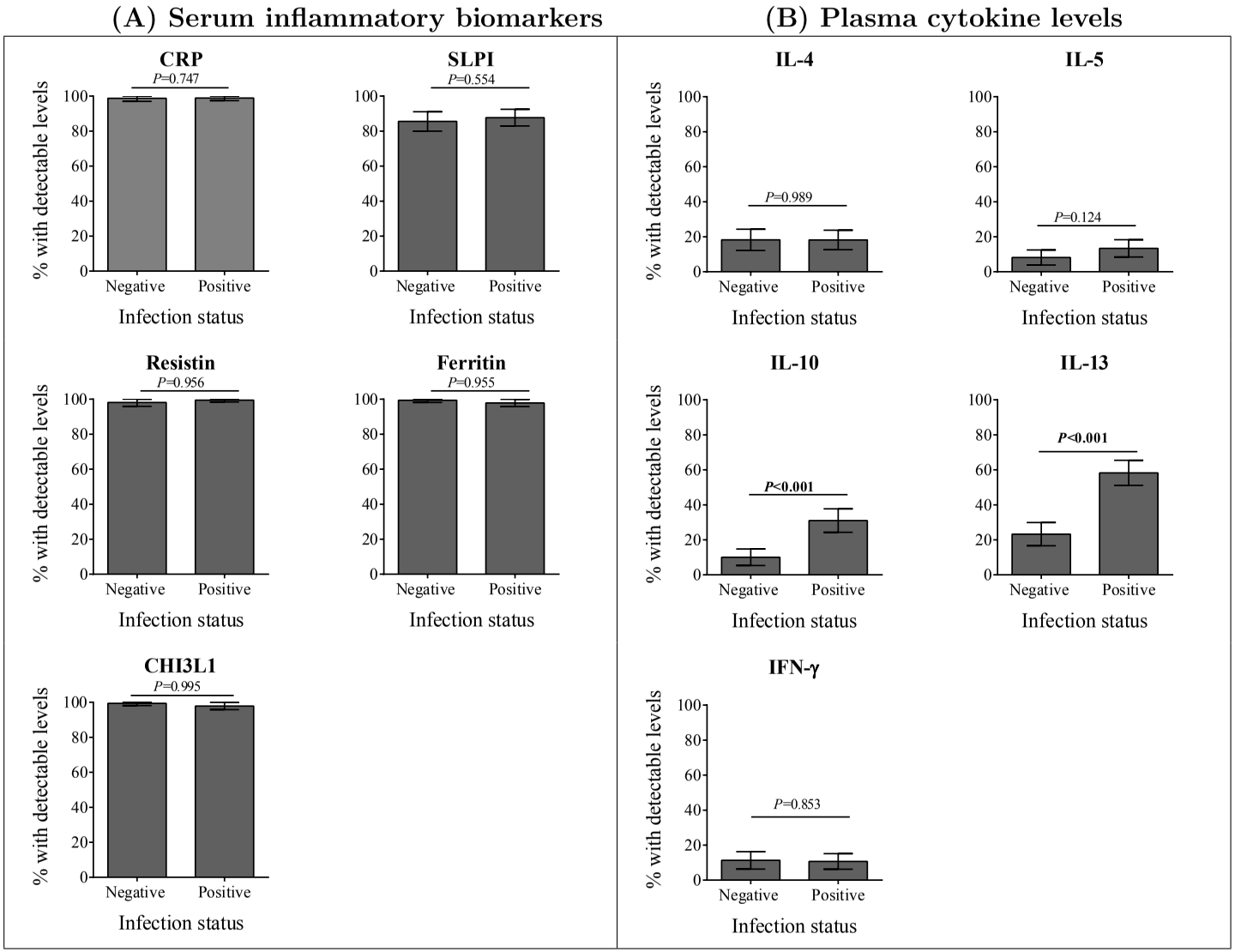


Figure F.2: Percentage frequency of children with detectable levels of biomarkers. Serum and plasma circulating levels measured by ELISA in children positive or negative for *S. haematobium* infection, as determined by a combination of parasitological and serological diagnostic techniques. Error bars indicate the 95% confidence intervals. *P*-values are from Chi-square (χ^2) tests for comparisons between infected and uninfected children. Significant differences are denoted in bold.

F.4 Model building steps

Table F.3: Model selection criteria based on mean square error (MSE). Comparing models with two-way interaction terms to the main effects model. Adding the interaction terms using the stepwise procedure did not result in a decrease in the MSEs of the respective models. The main effects models were selected as the best choice for interpretations. Infection=*S. haematobium* infection status

		Mean Square Error (MSE)				
Model	**Morbidity=Microhaematuria**	**CHI3L1**	**CRP**	**Ferritin**	**Resistin**	**SLPI**
(1)	Sex + Agegroup + Infection_status + Morbidity	0.0984	0.1778	0.0162	0.0508	1.0373
(2)	Sex + Agegroup + Infection + Morbidity + Sex*Agegroup	0.0985	0.1768	0.0148	0.0514	1.0388
(3)	Sex + Agegroup + Infection + Morbidity + Sex*Infection	0.0987	0.1781	0.0163	0.0516	1.0385
(4)	Sex + Agegroup + Infection + Morbidity + Sex*Morbidity	0.0987	0.1722	0.0163	0.0516	1.0400
(5)	Sex + Agegroup + Infection + Morbidity + Agegroup*Infection	0.0982	0.1758	0.0163	0.0505	1.0466
(6)	Sex + Agegroup + Infection + Morbidity + Agegroup*Morbidity	0.0985	0.1746	0.0162	0.0493	1.0310
(7)	Sex + Agegroup + Infection + Morbidity + Infection*Morbidity	0.0984	0.1784	0.0163	0.0509	1.0407
Model	**Morbidity=Proteinuria**					
(1)	Sex+Agegroup+Infection+Morbidity	0.0981	0.1788	0.0159	0.0506	1.0418
(2)	Sex + Agegroup + Infection + Morbidity + Sex*Agegroup	0.0981	0.1772	0.0160	0.0508	1.0431
(3)	Sex + Agegroup + Infection + Morbidity + Sex*Infection	0.0984	0.1791	0.0159	0.0508	1.0432
(4)	Sex + Agegroup + Infection + Morbidity + Sex*Morbidity	0.0978	0.1790	0.0160	0.0503	1.0450
(5)	Sex + Agegroup + Infection + Morbidity + Agegroup*Infection	0.0980	0.1764	0.0160	0.0503	1.0511
(6)	Sex + Agegroup + Infection + Morbidity + Agegroup*Morbidity	0.0970	0.1797	0.0159	0.0507	1.0464
(7)	Sex + Agegroup + Infection + Morbidity + Infection*Morbidity	0.0984	0.1794	0.0160	0.0505	1.0438
Model	**Morbidity=Albuminuria**					
(1)	Sex + Agegroup + Infection + Morbidity	0.0943	0.1655	0.0147	0.0512	1.1026
(2)	Sex + Agegroup + Infection + Morbidity + Sex*Agegroup	0.0940	0.1656	0.0147	0.0509	1.0956
(3)	Sex + Agegroup + Infection + Morbidity + Sex*Infection	0.0947	0.1660	0.0146	0.0513	1.1066
(4)	Sex + Agegroup + Infection + Morbidity + Sex*Morbidity	0.0943	0.1661	0.0145	0.0510	1.1038
(5)	Sex + Agegroup + Infection + Morbidity + Agegroup*Infection	0.0940	0.1643	0.0148	0.0511	1.1103
(6)	Sex + Agegroup + Infection + Morbidity + Agegroup*Morbidity	0.0943	0.1656	0.0147	0.0510	1.1072
(7)	Sex + Agegroup + Infection + Morbidity + Infection*Morbidity	0.0931	0.1661	0.0147	0.0511	1.0954

Table F.4: Relationship between plasma cytokine levels and presence of schistosome-related morbidity.

	IL-4		IL-5		IL-10		IL-13		IFN-γ		PC1		PC2	
Variable	**F**	**P**	**F**	**P**	**F**	**P**	**F**	**P**	**F**	**P**	**F**	**P**	**F**	**P**
(A)														
Sex	0.00	0.956	0.00	0.958	0.88	0.350	0.72	0.399	0.06	0.807	0.45	0.502	1.65	0.202
Age group	0.6	0.615	0.16	0.923	0.83	0.477	0.48	0.698	0.36	0.779	0.23	0.872	1.47	0.228
Infection status	3.32	0.070	4.22	0.041	0.12	0.729	0.15	0.699	0.55	0.459	0.05	0.819	2.16	0.145
Microhaematuria	0.23	0.632	2.27	0.133	3.24	0.074	1.97	0.163	0.47	0.492	0.19	0.661	1.65	0.202
(B)														
Sex	0.00	0.956	0.00	0.958	0.86	0.356	0.71	0.399	0.06	0.805	0.47	0.496	1.65	0.202
Age group	0.62	0.605	0.16	0.924	0.82	0.487	0.48	0.699	0.37	0.774	0.24	0.868	1.47	0.228
Infection status	3.41	0.067	**4.18**	**0.043**	0.12	0.732	0.15	0.699	0.56	0.455	0.05	0.817	2.16	0.145
Proteinuria	**4.95**	**0.027**	0.3	0.587	0.23	0.630	1.76	0.187	3.64	0.058	2.56	0.113	1.65	0.201
(C)														
Sex	0.00	0.952	0.24	0.623	0.86	0.355	0.71	0.402	0.33	0.564	0.45	0.50	1.63	0.205
Age group	0.81	0.492	0.92	0.434	0.82	0.485	0.47	0.703	0.71	0.548	0.23	0.873	1.45	0.233
Infection status	2.33	0.129	1.91	0.169	0.12	0.731	0.15	0.701	0.00	0.964	0.05	0.819	2.13	0.148
Albuminuria	2.14	0.146	1.64	0.202	0.83	0.365	0.09	0.769	1.22	0.270	0.02	0.876	0.42	0.519

Infection= Infection status determined by a combination of parasitological and serological diagnostic test results. PC1 (IL-4; IL-10; IFN-γ), PC2 (IL-13).

F.5 Subgroup analysis by *S. haematobium* egg status

Table F.5: Percentage of children with detectable levels of inflammatory biomarkers and cytokines by *S. haematobium* egg status. For each variable the percentage with detectable levels was compared between egg-negative (n=236) and egg-positive (n=96) children by Chi-square (χ^2) tests. Significant differences are highlighted and denoted in bold.

	% of children with detectable levels		
Variable	**Egg-negative**	**Egg-positive**	χ^2 (*P*-value)
Inflammatory biomarker			
CHI3L1	99.2 (98.0–100)	96.9 (93.3–100)	2.38 (0.122)
CRP	98.7 (97.3–100)	99.0 (96.9–100)	0.03 (0.999)
Ferritin	98.7 (97.3–100)	97.9 (95.0–100)	0.30 (0.629)
Resistin	98.3 (96.6–100)	100.0	*
SLPI	85.2 (80.6–89.7)	91.7 (86–97.3)	2.56 (0.110)
Cytokine response			
IL-4	18.6 (13.6–23.6)	15.6 (8.2–23.0)	0.42 (0.514)
IL-5	9.3 (5.6–13.1)	13.5 (6.6–20.5)	1.29 (0.256)
IL-10	12.7 (8.4–17)	41.7 (31.6–51.7)	**34.38 (<0.001)**
IL-13	28.8 (23–34.6)	74.0 (65.0–82.9)	**57.14 (<0.001)**
IFN-γ	11.4 (7.4–15.5)	8.3 (2.7–14.0)	0.70 (0.403)

**Test comparisons not determined.*

F.6 Adjusted means of inflammatory biomarkers in egg-positive children

Table F.6: Adjusted means of inflammatory serum biomarkers of *S.haematobium* egg-positive children (n=96) at baseline by schistosome-related urinary morbidity markers (negative or positive). The log-transformed mean levels (ng/mL) were adjusted for sex and age group. The *P*-values were obtained from post-hoc tests for general linear models.

	Mean ± SE		Mean difference		
	Negative	**Positive**	**(95% CI)**	***t***	P^{1}
(A) Microhaematuria					
CHI3L1	1.88 ± 0.27	1.42 ± 0.09	0.46 (-0.04–0.96)	1.83	0.071
CRP	2.45 ± 0.37	2.45 ± 0.13	0.00 (-0.70–0.70)	0.01	0.991
Ferritin	0.31 ± 0.10	0.32 ± 0.03	-0.01 (-0.19–0.17)	-0.16	0.873
Resistin	0.78 ± 0.18	0.62 ± 0.06	0.16 (-0.18–0.49)	0.93	0.353
SLPI	3.41 ± 0.69	2.71 ± 0.24	0.70 (-0.59–1.99)	1.08	0.282
(B) Proteinuria					
CHI3L1	1.43 ± 0.10	1.42 ± 0.11	0.01 (-0.14–0.16)	0.11	0.914
CRP	2.47 ± 0.14	2.40 ± 0.15	0.06 (-0.15–0.27)	0.58	0.561
Ferritin	0.34 ± 0.03	0.29 ± 0.04	0.05 (-0.01–0.10)	1.78	0.078
Resistin	0.64 ± 0.06	0.58 ± 0.07	0.06 (-0.04–0.16)	1.27	0.206
SLPI	2.73 ± 0.25	2.70 ±0.28	0.02 (-0.36–0.41)	0.12	0.901
(C) Albuminuria					
CHI3L1	1.36 ± 0.10	1.47 ± 0.10	-0.10 (-0.26–0.05)	-1.30	0.197
CRP	2.39 ± 0.14	2.49 ± 0.14	-0.09 (-0.31–0.12)	-0.84	0.405
Ferritin	0.33 ± 0.04	0.31 ± 0.04	0.02 (-0.04–0.07)	0.54	0.594
Resistin	0.60 ± 0.07	0.65 ± 0.08	-0.04 (-0.16–0.06)	-0.93	0.357
SLPI	2.55 ± 2.03	2.86 ± 2.34	-0.31 (-0.71–0.10)	-1.51	0.135

SE=standard error of the mean.
[1] *P-values adjusted for multiple comparisons using the Bonferroni correction.*

F.7 Post-treatment CHI3L1 and CRP levels in egg-positive children

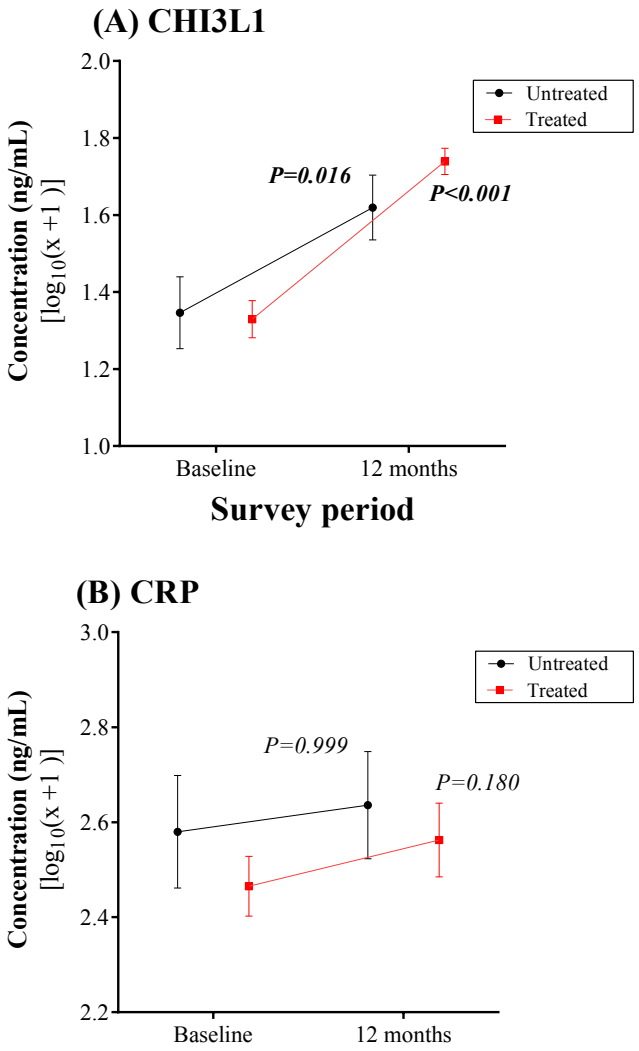


Figure F.3: Mean serum levels of CHI3L1 and CRP levels at baseline and 12 months after treatment in children diagnosed egg-positive at baseline. Error bars indicate the standard error of the mean. Tests for the change from baseline in mean levels of biomarkers 12 months post-treatment†.

†Due to the small sample size of the untreated group, the analyses were performed using the Wilcoxon Signed Rank Test.

Appendix G

Survey Questionnaires

- Questionnaires for the preschool-aged children (1–5 years), answered on their behalf by parents or guardians.
- Questionnaires for the primary school-aged children (6–10 years).

Questionnaire
Bilharzia in Pre-School Children

This questionnaire is 6 pages long- please complete ALL pages.

Questionnaire to be completed at the time of visit with parents and enrollment of children into the study and only after obtaining parents consent and assent of participating child.

Questions are all to be answered by the adult who brings the child for enrollment

Date of questionnaire _____

Person administering questionnaire _____

Name of adult accompanying child _____

Relationship of named adult to child _____

Study ID# for named adult _____

The following details/ questions are about the CHILD.

Name	
Study ID	
Date of birth	
Age	
Sex	
Height (cm)	
Weight (kg)	
Arm circumference (cm)	
Head circumference	
Body temperature	
Village of residence	

Has the child lived in this village all their life? Yes No ***(circle either yes or no)***

If no, **How long has the child lived in this village?** _____ (years)

QUESTIONS

1. **Does the child have brothers or sisters?** Yes No ***(circle either yes or no)***

If yes, how many older? 1 2 3 more ***(circle one)***

How many are in school? 1 2 3 more ***(circle one)***

Please give name, school and grade for all brothers/sisters at school:

Name	**School**	**Grade**
_____	_____	_____
_____	_____	_____
_____	_____	_____
_____	_____	_____

2. Has the child ever had Bilharzia? Yes No Do not know ***(circle one)***

3. Has the child ever been given treatment for Bilharzia? Yes No Do not know ***(circle one)***

If yes, do you remember When? _____(year)

Where? _____

How? Tablets / liquid/ other ***(circle one)***

4. Have the child's brothers/sisters ever had Bilhar Yes No Do not know ***(circle one)***

5. Have the child's brothers/sisters ever been give treatment for Bilharzia? Yes No Do not know ***(circle one)***

If yes, do you remember When? _____(year)

Where? _____

How? Tablets / liquid/ other ***(circle one)***

6. Has the child ever had Malaria? Yes No Do not know ***(circle one)***

7. Has the child ever been given treatment for Malaria? Yes No Do not know ***(circle one)***

If yes, do you remember When? _____(year)

Where? _____

How? Tablets / liquid/ other ***(circle one)***

8. Have the child's brothers/sisters ever had Malar Yes No Do not know ***(circle one)***

9. Have the child's brothers/sisters ever been give treatment for Malaria? Yes No Do not know ***(circle one)***

If yes, do you remember When? _____(year)

Where? _____

How? Tablets / liquid/ other ***(circle one)***

10. Has the child ever had:

a) **Blood in their urine?** Yes No Do not know ***(circle one)***
b) **Difficulty in urinating?** Yes No Do not know ***(circle one)***
c) **Frequent urge to urinate?** Yes No Do not know ***(circle one)***
d) **Pain in their joints?** Yes No Do not know ***(circle one)***
e) **Diarrhoea?** Yes No Do not know ***(circle one)***

11. Does the child currently have:

a) **Blood in their urine?** Yes No Do not know ***(circle one)***
b) **Difficulty in urinating?** Yes No Do not know ***(circle one)***
c) **Frequent urge to urinate?** Yes No Do not know ***(circle one)***
d) **Pain in their joints?** Yes No Do not know ***(circle one)***
e) **Diarrhoea?** Yes No Do not know ***(circle one)***
f) **Other symptoms (specify)** _____

12. Has the child ever been to hospital since birth (other than for vaccinations)? Yes No ***(circle either yes or no)***

If yes, what for? _____

when? _____

(You do not have to disclose any results of tests undertaken)

13. Has the child received vaccination for:

a.	BCG vaccination (tuberculosis)?	Yes	No	Don't know	***(circle one)***
b.	Mumps	Yes	No	Don't know	***(circle one)***
c.	Measles	Yes	No	Don't know	***(circle one)***
d.	Rubella	Yes	No	Don't know	***(circle one)***
e.	Diphtheria	Yes	No	Don't know	***(circle one)***
f.	others (specify) _____				

14. Where does your drinking water come from? *(circle as many as appropriate)*

a. Unprotected well
b. River
c. Dam
d. Upgraded well
e. Borehole
f. Tap
g. Other (specify) _____

15. Where does the child normally go to the toilet? *(circle as many as appropriate)*

a. Bush
b. cat sanitation
c. Latrine/toilet
d. Other (specify) _____

16. Is there a latrine at your home? Yes No ***(circle either yes or no)***

17. If yes, are there any problems in using it? Yes No ***(circle either yes or no)***

If yes, explain _____

18. Water contact questions:

	Y/N	**How often?** ***(tick √ one box - see scale below)***								**Where do they normally go? (name of river/dam)**	**What time of day do they normally do it?** (morning/ midday/ afternoon/evening)
Does the child ever play in the river or dam?											
Does the child ever bathe in the river or dam?											
Does the child ever collect water from the river or dam?											
Does the child ever cross any rivers?											
Does the child ever water the garden from the river or dam?											
Does the child ever go to the river or dam for any other reason? Reason _____											

19. How often does the child go to the river or dam?

Once a year | Once a month | Once a week | Once a day

20. Where does the child normally get treated when unwell? *(circle as many as appropriate)*
a. At home using herbal medicines
b. At home using western medicine
c. At the traditional healer
d. At the local clinic
e. Other please specify_____

21. Where do <u>you</u> normally get treated when unwell? *(circle as many as appropriate)*
a. At home using herbal medicines
b. At home using western medicine
c. At the traditional healer
d. At the local clinic
e. Other please specify_____

*[The following questions are simply to assess knowledge of the participants' mothers and are not to be used for inclusion / exclusion. Ask the question given in bold and circle the answers which best match their response. **DO NOT READ OUT THE LIST OF ANSWERS**]*

22. What is Bilharzia? *(circle one answer*)
a. Don't know
b. A disease
c. A disease transmitted by drinking dirty water
d. A disease transmitted by snails that live in rivers and ponds
e. A disease caused by tiny parasitic worms which live in rivers and ponds
f. A disease caused by tiny parasitic worms which are transmitted by snails that live in rivers and ponds
g. Other, please state_____

23. Where did you learn about Bilharzia? *(circle as many as appropriate*)
a. School
b. Health centre
c. Community
d. Family
e. Read book
f. Nowhere
g. Other _____

24. What are the main symptoms of Bilharzia? (*circle as many as appropriate*)
a. Don't know
b. Vomiting
c. Blood in urine
d. Pain when urinating
e. Frequent urination
f. Headache
g. Stomach ache
h. Tiredness
i. Loose and bloody stools
j. Other, please state_____

25. How do you catch Bilharzia? (*circle ONLY one answer which best describes their knowledge*)
a. Don't know
b. By being bitten by mosquitoes
c. By drinking dirty water
d. By playing or bathing in rivers or ponds where snails are present
e. By playing or bathing in rivers or ponds
f. Other please state_____

26. How can you protect yourself from Bilharzia? (*circle as many as appropriate*)
a. Don't know
b. By stopping to play or bathe in rivers and ponds
c. By you (and others) always using a latrine.
d. By taking medicines regularly
e. By removing freshwater snails
f. Other, please state_____

27. Have you ever taken treatment for Bilharzia? Yes No Do not know **(*circle one*)**

If yes, when? _____(year)

28. Can you ever catch Bilharzia again after taking treatment?

Yes No Do not know **(*circle one*)**

29. What are the main symptoms of malaria? (*circle as many as appropriate*)
a. Don't know
b. Tiredness
c. Vomiting
d. Headache
e. Diarrhoea
f. Fever and chills
g. Other, please state_____

29. How do you catch malaria? (***circle ONLY one answer which best describes their knowledge***)
a. Don't know
b. By drinking dirty water
c. By being bitten by mosquitoes that bite during day-time
d. By being bitten by mosquitoes that bite during night-time
e. Other, please state_____

30. How can you protect yourself from malaria? (***circle as many as appropriate***)
a. Don't know
b. By drinking clean water from the well
c. By taking medicines
d. By wearing insect repellents
e. By sleeping each night under a mosquito bed net
f. By regularly spraying insecticides inside the home
g. Other, please state_____

31. Do you know anyone who has had malaria?

Yes No **(*circle either yes or no*)**

If yes, who? _____

32. Have you ever taken treatment for malaria?

Yes No Do not know **(*circle one*)**

If yes, when? _____(year)

33. Can you ever catch malaria again after taking treatment?

Yes No Do not know **(*circle one*)**

COMMENTS FROM HEALTHWORKER EXAMINING CHILD

_____

_____

_____

_____

_____

_____

_____

_____

ANY OTHER COMMENTS

_____

_____

_____

_____

_____

_____

_____

_____

WT Bilharzia Questionnaire

1. **This questionnaire is 4 pages long- please complete ALL pages.**
2. **Questionnaire to be completed at the time of enrollment of children into the study and only after obtaining parents consent and assent of participating child.**

Date of questionnaire_____
Person administering questionnaire_____
Parent's name _____

Participant Name_____ **ID #**_____

Gender M F ***(circle one)***

Date of birth (DD MM YY)_____ **Age**_____(years)

Age group: 6-10 11-14 15-17 18-25 26-35 36+ ***(circle one)***

Body weight _____kg **Height** _____cm

School _____ **Village Name**_____

Have you lived in this village all your life? Y N **(*circle either yes or no*)**

If no, **which other villages have you lived in and for how long?**

Order of residence	Village name	District	How long (years)?
1 (born in)	_____	_____	_____
2	_____	_____	_____
3	_____	_____	_____
4	_____	_____	_____
5	_____	_____	_____

Which primary school did you attend? _____

Which secondary school did you attend? _____

Do you make regular visits to other villages? Y N ***(circle either yes or no*)**

If yes, Where? _____

When? _____

Do you participate in sports? Y N ***(circle either yes or no*)**

If no, why not? _____

Have you ever had Schistosomiasis (bilharzia) infection? Yes / No / Do not know ***(circle one)***

If yes, were you treated? Yes / No / Do not know ***(circle one)***

If yes, do you remember when you were treated?_____ How?_____

Have you ever had Malaria? Yes / No / Do not know ***(circle one)***

If yes, were you treated? Yes / No / Do not know ***(circle one)***

If yes, do you remember when you were treated?_____ How?_____

Other Signs and symptoms (ask then prompt from list)
Hematuria (Blood in urine) _____
Dysuria (Difficulty in urination)_____
Frequent urge to urinate (approximate estimate)_____
Fever I (37.5-38.5^{0}C) II (38.6-40^{0}C) III (40^{0}C+)
Chills_____
Arthralgia (Pain in joints) _____
Myalgia (Muscle pain) _____
Backache _____
Lethargy (Sleepy)_____
Diarrhea _____
Other symptoms_____
Splenomegaly (enlarged spleen) _____
Hepatomegaly (enlarged liver) _____
Hepatitis B antibody (if known)_____

Which village were you born in?_____
(check this matches village they live in now – if not check have full list of all villages lived in)

The following information is being collected to assess various likely or probable risk factors associated with malaria and / or schistosomiasis infection

A. Domestic water source ***(circle as many as appropriate)***

1 unprotected well
2 river
3 dam
4 Upgraded well
5 Borehole
6 Tap
7 Other (specify) _____

B. Where do you normally go to the toilet? ***(circle as many as appropriate)***

1 bush
2 cat sanitation
3 Latrine/toilet
4 Other (specify) _____

C. Is there a latrine at your home? Y N ***(circle either yes or no)***

D. If yes, are there any problems in using it? Y N ***(circle either yes or no)***

If yes, explain _____

E. Do you pass through water on your way to and from school? Y N ***(circle one)***

If yes, where? (describe and/or give site name)_____

F. Water contact activities

	Yes/No	(code)	How many times?	Where?* River/Dam/Well/Other (if Other, specify)	(code)	If River or Dam, name of place or nearest village/school	(code)	At what time?* (e.g. 0800, 1200, 1600)	(code)
(1) Did you go swimming yesterday?									
(2) Did you play in the water yesterday?									
(3) Did you go to bathe yesterday?									
(4) Did you do laundry yesterday?									
(5) Did you wash dishes yesterday?									
(6) Did you go to wash yesterday? (face and legs)									
(7) Did you collect water yesterday?									
(8) Did you go fishing yesterday?									
(9) Did you cross any rivers yesterday?									
(10) Did you go to the water for any other reason yesterday?									
(10 a) If answer to Q10 **yes**: What did you do?_____									

***Answer for each contact**

G. In the last 7 days, have you carried out any of these activities at the river or dam? *(circle as many as appropriate)*

	If yes, how many times?		If yes, how many times?
Swimming	_____	Washing (face and legs)	_____
Playing in the water	_____	Collecting water	_____
Bathing	_____	Fishing	_____
Laundry	_____	Crossing river	_____
Washing dishes	_____	Other_____	_____

The following questions are simply to assess knowledge of the participants and are not to be used for inclusion / exclusion)

H. Do you know what bilharzia is? Y N ***(circle either yes or no*)**

If yes, where did you learn about bilharzia? *(circle as many as appropriate*)

1. School
2. Health centre
3. Community
4. Family
5. Read book
6. Nowhere
7. Other _____

I. Do you know what the symptoms of bilharzia are? *(circle as many as appropriate*)

1. Blood in urine
2. Tiredness
3. Mental illness
4. Pain upon urination
5. Other _____

J. Do you know how bilharzia is spread? *(circle as many as appropriate*)

1. Urinating in water
2. Defecating in water
3. Swimming
4. Stepping on urine
5. Drinking dirty water
6. Snails
7. Other _____

K. Do you know what the long-term effects of bilharzia are? *(circle as many as appropriate*)

1. Infertility
2. Stomach/bladder damage
3. Death
4. Going mad
5. Don't know
6. Other _____

L. Do you try to protect yourself from getting bilharzia? Y N ***(circle one*)**

If yes, how? _____

If no, why not? *(circle as many as appropriate*)

Economic reasons
Distance to clinic
Lack of knowledge of treatment options,
Religious reasons
Other _____

M. Do you have any questions or comments concerning this study?

Appendix H

Publications of work related to the thesis

- Poster presented at the British Society for Parasitology Autumn Symposium 2013 on "Advances in Diagnostics for Infectious Diseases".
- Comparing parasitological *vs.* serological determination of *Schistosoma haematobium* infection prevalence in preschool and primary school-aged children: implications for control programmes. In *Parasitology*, 2014.
- Identifying and Evaluating Field Indicators of Urogenital Schistosomiasis-Related Morbidity in Preschool-Aged Children. In *PloS NTD*, 2015.

UROGENITAL SCHISTOSOMIASIS MORBIDITY IN PRE-AND PRIMARY SCHOOL-AGED CHILDREN IN RURAL ZIMBABWE

W.M. Wami[1]*, N. Nausch[1], N. Midzi[2], T. Mduluza[3], M.E.J. Woolhouse[4], F. Mutapi[1]

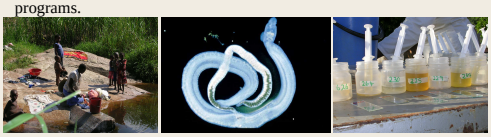
[1] Institute for Immunology & Infection Research, Ashworth Laboratories, University of Edinburgh, Edinburgh EH9 3JT, UK
[2] National Institute of Health Research, PO Box CY 573, Harare, Zimbabwe, [3] Department of Biochemistry, University of Zimbabwe, PO Box 167, Harare, Zimbabwe
[4] Center for Immunity, Infection & Evolution, Ashworth Laboratories, University of Edinburgh, Edinburgh EH9 3JT, UK
*w.m.wami@sms.ed.ac.uk

THE UNIVERSITY of EDINBURGH

THRASHER RESEARCH FUND

Introduction

- Urogenital schistosomiasis caused by *Schistosoma haematobium*, affects 119 million people worldwide.
- Infection occurs on contact with infested waters and in endemic regions re-infection is widespread.
- Treatment by praziquantel (PZQ), 40mg/kg single dosage.
- Pre-school children (≤5 years) still not targeted by treatment control programs.



Water contacts, Male and Female Schistosomes and Urine samples.

Aim

The aim of this study was to assess urogenital schistosomiasis morbidity prevalence in children residing in an endemic region and effect of PZQ treatment 6 weeks post-treatment on infection levels.

Methods

Study area and Population

- Murehwa district, NE Zimbabwe (*S. haematobium* endemic).
- Pre- and primary school children aged 1-10 years, lifelong residents.
- No previous/current geo-helminths infection.
- No previous history of anti-helminthic treatment.

Study Area (red star) and Study population

Treatment

- Single dosage of PZQ in tablet form with 6 weeks post-treatment efficacy follow-up.

Parasitology and Morbidity

- 10ml urine samples collected on 3 consecutive days.
- Egg counts/10ml urine determined using urine filtration method.
- Reagent strip used to test for morbidity: Haematuria, Proteinuria and Urine-Albumin/Creatine Ratio (UACR).

Statistical Analysis

To investigate whether PZQ treatment efficacy is associated with age, a general linear model (GLM) was fitted with infection intensity at 6 weeks post-treatment as response variable, adjusting for pre-treatment infection intensity. A model with an interaction term between age and pre-treatment infection intensity was first considered. Level of significance was set at $\alpha = 0.05$.

Results

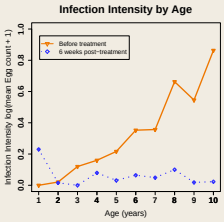
Summary Statistics at Baseline (Pre-treatment).

Variable	Parameter	1-5 years	6-10 years	Overall
Sample	n	146	268	414
Sex (%)	Male	53.8	42.5	46.5
	Female	46.2	57.5	53.5
Age	mean(SD)	3 (1.4)	8 (1.4)	6 (2.9)
Egg count	mean(SD)	3 (18.3)	21 (81.3)	15 (67.6)
	Minimum	0	0	0
	Maximum	189	1013	1013

*Egg count per 10ml urine; SD=standard deviation.

- Presence of heavy infections (egg count≥50) among children.

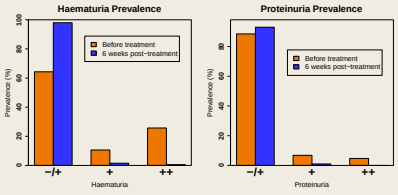
Infection intensity, Egg reduction rate (ERR) and Cure Rate (CR).

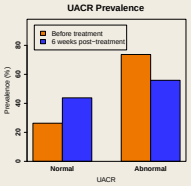


Age group	ERR (95%CI)	CR (95% CI)
1-5 years	86.2 (32.4-98.8)	91.7 (64.6-98.5)
6-10 years	95.8 (77.3-99.2)	92.1 (84.6-96.1)
Overall	93.3 (70.2-98.8)	92.1 (85.1-95.9)

- Infection intensity higher in the older children pre-treatment.
- High efficacy in both age groups.

Prevalence of Haematuria, Proteinuria and UACR.





- Decrease in prevalence of morbidity post-treatment.
- Abnormal levels of UACR observed 6 weeks post-treatment.

Treatment Efficacy: Is there an age effect?.

- Interaction effect was not significant, hence reduced.
- No significant association between post-treatment infection intensity and age (t=-1.3; p-val=0.18), adjusting for pre-treatment infection intensity (t=1.5; p-val=0.13).

Conclusion

- **Heavy infection intensity and high morbidity prevalences were noted in this young population pre-treatment.**
- **Treatment reduces/clears infection and reverses morbidity.**
- **PZQ treatment efficacy high and is not age related.**
- **There is need for control programs to target younger children and important to consider re-treatments to target immature infections.**

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Comparing parasitological *vs* serological determination of *Schistosoma haematobium* infection prevalence in preschool and primary school-aged children: implications for control programmes

WELCOME M. WAMI[1,4]*, NORMAN NAUSCH[1,4], KATHARINA BAUER[1,4]†, NICHOLAS MIDZI[2]‡, REGGIS GWISAI[3], PETER SIMMONDS[4], TAKAFIRA MDULUZA[5], MARK WOOLHOUSE[4] *and* FRANCISCA MUTAPI[1,4]

[1] *Institute of Immunology & Infection Research, University of Edinburgh, Ashworth Laboratories, King's Buildings, West Mains Rd, Edinburgh EH9 3JT, UK*
[2] *National Institute of Health Research, P.O. Box CY 573, Causeway, Harare, Zimbabwe*
[3] *Ministry of Health and Child Care, Murewa District Hospital, P.O. Box 60, Murewa, Zimbabwe*
[4] *Centre for Immunity, Infection & Evolution, University of Edinburgh, Ashworth Laboratories, King's Buildings, West Mains Rd, Edinburgh EH9 3JT, UK*
[5] *University of Zimbabwe, Biochemistry Department, P.O. Box MP167, Mount Pleasant, Harare, Zimbabwe*

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SUMMARY

To combat schistosomiasis, the World Health Organization (WHO) recommends that infection levels are determined prior to designing and implementing control programmes, as the treatment regimens depend on the population infection prevalence. However, the sensitivity of the parasitological infection diagnostic method is less reliable when infection levels are low. The aim of this study was to compare levels of *Schistosoma haematobium* infection obtained by the parasitological method *vs* serological technique. Infection levels in preschool and primary school-aged children and their implications for control programmes were also investigated. Infection prevalence based on serology was significantly higher compared with that based on parasitology for both age groups. The difference between infection levels obtained using the two methods increased with age. Consequentially, in line with the WHO guidelines, the serological method suggested a more frequent treatment regimen for this population compared with that implied by the parasitological method. These findings highlighted the presence of infection in children aged $\leqslant 5$ years, further reiterating the need for their inclusion in control programmes. Furthermore, this study demonstrated the importance of using sensitive diagnostic methods as this has implications on the required intervention controls for the population.

Key words: parasitology, serology, prevalence, schistosomiasis, diagnosis, neglected tropical diseases.

INTRODUCTION

Urogenital schistosomiasis is a waterborne disease caused by infection with *Schistosoma haematobium* and is a major public health problem among poor communities in sub-Saharan Africa (Gryseels *et al*. 2006; Kabatereine *et al*. 2007; WHO, 2012). Eggs laid by adult female *S. haematobium* worms are excreted through urine, inflicting damage to the genitourinary tract. Children living in endemic areas tend to carry the highest disease burden (Hotez *et al*. 2006; Stothard *et al*. 2011*a*) and symptoms of urogenital schistosomiasis amongst these children are commonly characterized by the presence of blood in urine (haematuria) and painful urination (van der Werf *et al*. 2003; Sady *et al*. 2013). Chronic infection results in severe pathologies such as kidney failure and urinary tract and bladder wall fibrosis. Other symptoms include malnutrition, stunted growth and impaired memory and cognition (Pasvol and Hoffman, 2001; Sousa-Figueiredo *et al*. 2008; WHO, 2010; Muller *et al*. 2011).

The infection and its associated morbidity can be controlled with chemotherapy using praziquantel (Doenhoff *et al*. 2008; Mutapi *et al*. 2011), administered at a standard oral dosage of 40 mg kg^{-1} body weight (WHO, 2002). Praziquantel is safe and efficacious in children aged 5 years or under (Mutapi *et al*. 2011; Stothard *et al*. 2011*b*; Coulibaly *et al*. 2012), but so far treatment of children belonging to

* Corresponding author: Institute of Immunology & Infection Research, School of Biological Sciences, University of Edinburgh, Ashworth, Laboratories, King's Buildings, West Mains Rd, Edinburgh EH9 3JT, UK. E-mail: W.M.Wami@sms.ed.ac.uk
† Current address: German Cancer Research Center (DKFZ), Im Neuenheimer Feld 280, 69120 Heidelberg, Germany.
‡ Current address: University of Zimbabwe, College of Health Sciences, Department of Medical, Microbiology, P.O. Box A178, Avondale, Harare, Zimbabwe.

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this age group has not yet been fully integrated into the control programmes (Ekpo *et al*. 2012). Preschool-aged (⩽ 5 years) children have been neglected both in terms of research and in control programmes for the previously held view that they carry insignificant infection levels (Stothard and Gabrielli, 2007; Mutapi *et al*. 2011; WHO, 2011). This was further exacerbated by poor diagnosis of infection in the field (Vennervald *et al*. 2000; Stothard *et al*. 2011*a*). The exclusion of preschool-aged children from current control programmes increases their risk of developing future morbidity (Stothard and Gabrielli, 2007; Sousa-Figueiredo *et al*. 2008) and also indicates that disease burden in this age group is still not well defined (Garba *et al*. 2010). Consequently, this may have negative impacts on the overall effectiveness of control programmes.

In line with the guidelines outlined by the World Health Organization (WHO, 2002), infection prevalence must be determined prior to the implementation of a control programme (Dawson *et al*. 2013; WHO, 2013). To ensure that infection transmission levels are reduced and the associated morbidity is alleviated, repeated mass drug administration (MDA) at regular intervals depending on the population prevalence has been recommended by the WHO (Hotez *et al*. 2006; Kabatereine *et al*. 2007; WHO, 2013). Thus, it is important that sensitive diagnostic tools are applied to determine infection levels in the population.

Egg count in urine (parasitology) is the widely accepted approach for quantifying *S. haematobium* infection levels in a population (WHO, 1998; Pasvol and Hoffman, 2001; Kinkel *et al*. 2012). However, the parasitological method is less sensitive in light infections (Doenhoff *et al*. 2004; Bergquist *et al*. 2009). Furthermore, parasitology does not diagnose pre-patent or single-sex infections where there is no egg production (Mutapi, 2011). Several additional methods aimed at improving the diagnosis of schistosomiasis have been evaluated (Stothard *et al*. 2013), although the focus has been mainly on *Schistosoma mansoni* (Sorgho *et al*. 2005; de Noya *et al*. 2007; Stothard *et al*. 2011*a*). Examples of additional diagnostic methods include antibody detection (Sorgho *et al*. 2005; de Noya *et al*. 2007; Smith *et al*. 2012), dipstick detection of haematuria (Adesola *et al*. 2012; King and Bertsch, 2013) and use of reported questionnaires about presence of haematuria (Lengeler *et al*. 2002; Clements *et al*. 2008). There is currently a paucity of studies comparing different methods of detecting infection in preschool-aged children. The elegant dipstick meta-analysis study recently published by King and Bertsch (2013) highlights the need for more investigations on different methods for detecting infection in preschool-aged children.

The first aim of our study was to compare levels of *S. haematobium* infection determined by the parasitological method with infection detected via the serological technique and their implications for the WHO recommended treatment regimens for this study population. Dipstick microhaematuria was also used as an additional tool to the parasitological method on a subset of this study population to detect *S. haematobium* infection. The second aim of this study was to determine infection levels in preschool-aged children in comparison to primary school-aged children to elucidate the implications of these levels of infection for childhood health and their inclusion in the current control programmes.

MATERIALS AND METHODS

Ethical approval and consent

The study received ethical and institutional approval from the University of Zimbabwe and the Research Council of Zimbabwe. Permission to conduct the work in this province was obtained from the Provincial Medical Director, the District Educational Officer and Heads of schools in the study area. Project aims and procedures were fully explained to the community, primary school-aged children, teachers and parents/guardians in the local language, Shona. Written informed consent/assent was obtained from parents/guardians prior to enrolment of children into the study. The children were recruited into the study on a voluntary basis and were free to withdraw at any time with no further obligation. Children in this study were offered treatment with the standard dose of praziquantel administered by the local physician.

Study area and population

The study was conducted in two rural villages in Murewa district, in the north-east of Zimbabwe (31°90′E; 17°63′S). The area is a high *S. haematobium* transmission area according to the WHO classification of having a prevalence of infection >50% (WHO, 2002). Prevalence of *S. mansoni* and soil transmitted helminths (STH) is low in this area (Ndhlovu *et al*. 1996; Nausch *et al*. 2012). The children were recruited from crèches, early child development centres, preschools (typically for 3–5 years old) and local primary schools (for 6–10 years old). Parents/guardians with children not attending any of the education programmes (e.g. children <3 years old) in the area were invited to report to the school centre for enrolment into the project.

Study design

The inclusion/exclusion criteria for this study were as follows: children should have (1) been lifelong residents of the study area; (2) had no prior history of anthelmintic treatment (the above two criteria were

assessed by means of questionnaires administered to parents/guardians for all children); (3) had provided at least 3 urine samples for *S. haematobium* detection and 2 stool samples for STH and *S. mansoni* parasitological examination; (4) been negative for *S. mansoni* infection (21 children were excluded from the study based on this criterion); and (5) been negative for STH infections (no children were excluded based on this criteria as no STH were detected in any of the participants). A total of 438 children (54·6% females and 48·9% males) with complete parasitological and serological data were available for investigation in this study (Table A1). Of the surveyed children, 224 (51·1%) resided in village 1 and 214 (48·9%) were residents of village 2.

Parasitology

Urine samples collected on 3 consecutive days were examined microscopically for *S. haematobium* infection using the standard filtration method (Mott *et al.* 1982). *Schistosoma mansoni* infection was diagnosed from stool samples collected on 2 consecutive days using the Kato-Katz method (Katz *et al.* 1972). Children were designated infected with *S. haematobium* if at least one egg was detected in any of their urine samples and similarly for *S. mansoni* with a single egg detected in stool. The *S. haematobium* infection intensity was calculated using the arithmetic mean egg count per 10 mL of the collected urine samples. For very young children where it was difficult to obtain samples on the spot, the samples were collected overnight by parents/guardians using urine collection bags (Hollister 7511 U-Bag Urine Specimen Collector, Hollister Inc., Chicago, IL, USA) and stool samples were collected using disposal dippers.

Serology

Serum was obtained from up to 5 mL of venous blood collected from each child, frozen at −20 °C in the field and transferred to a −80 °C freezer in the laboratory, prior to shipment to the University of Edinburgh, UK and kept under storage at −80 °C. Samples were thawed for the first time for use in this study. The sera were tested for IgM (Dako, UK) antibody responses directed against schistosome egg antigens using enzyme linked immunosorbent assays (ELISA). The ELISA were conducted in duplicate per plate as previously described (Mutapi *et al.* 1997; Imai *et al.* 2011). The results were expressed as the mean optical density (OD) value of the duplicate assay. IgM antibodies are produced early in an infection (Warrington *et al.* 2011) and previous studies have reported a positive association between anti-egg IgM antibody responses and schistosomiasis infection levels (Mutapi *et al.* 2003; Stothard *et al.* 2011*a*; Dawson *et al.* 2013). Thus, for this study we used anti-schistosome egg IgM antibody response as an additional diagnostic indicator for *S. haematobium* infection.

A total of 17 serum samples comprised of four serum samples from age-matched schistosome naïve European and 13 healthy Zimbabwean donors (schistosome infection-free and with no anomalies reported after clinical examination by the paediatrician) were used as controls to determine cut-off ELISA values for 'infection' status. The European samples were drawn from the Edinburgh anonymized clinical sample archive. The cut-offs were calculated using the formula: mean (OD) +2*standard deviations of the mean (S.D.). Children were classified as infected if their levels of parasite-specific antibody levels were greater than the cut-off value, and infection negative if equal or below the cut-off value.

Dipstick microhaematuria

Out of the 438 children, 190 (51 preschool-aged and 139 primary school-aged) children in this study population had their urine samples examined for microhaematuria detectable by Uristix® reagent strips (Plasmatec, UK) as an indicator for *S. haematobium* infection in addition to the parasitological and serological diagnostic methods (Table A2). No marked variability was noted in dipstick tests of the urine samples for each child collected on consecutive days, thus, only the dipstick test results for urine samples collected on the first day of the survey were used in this study. The levels of dipstick microhaematuria were first graded semi-quantitatively as: negative (−), single positive (+; $\approx$10 erythrocytes μL^{-1}), double positive (++; $\approx$50 erythrocytes μL^{-1}) and strong positive (+++; $\approx$250 erythrocytes μL^{-1}) following the manufacturer's guidelines. In this study, a positive test for microhaematuria was indicative of the presence of *S. haematobium* infection, meaning that children with scores of a single + and above were scored as positive for microhaematuria. A random sample of 123 urine samples were tested using the Multistix® 10SG (Bayer, UK) in addition to the Uristix® test to assess for differences in the quality of dipsticks by manufacturer. A strong agreement between the dipstick results from the two manufacturers using the McNemar's test ($P<0{\cdot}001$) was observed, hence no evidence of the influence of the dipstick source on test results was noted for this study population.

Statistical analyses

Statistical analyses were performed using SAS® 9.3 (SAS Institute Inc., Cary, NC, USA) and R 3.0.1 (R Development Core Team, Vienna, Austria). Infection intensity was log-transformed ($\log_{10}$[egg

Table 1. Summary results for infection intensity using egg count per 10 mL urine, IgM antibody response in optical densities (OD) directed against schistosome egg antigens with standard deviation of the mean (S.D.) and *t*-test (on transformed data for infection intensity) for mean difference between the two age groups

Variable	Age group	*n*	Mean (S.D.)	Median	Minimum	Maximum	*t*	*P* value
Egg count	1–5 years	97	9·03 (47·53)	0·00	0·00	380·33	−4·49	<0·001
	6–10 years	341	19·78 (76·50)	0·00	0·00	1013·00		
Antibody level	1–5 years	97	0·46 (0·31)	0·38	0·01	1·27	−5·72	<0·001
	6–10 years	341	0·67 (0·33)	0·66	0·07	2·39		

count +1]) to meet the underlying assumptions of parametric statistical tests. Pearson's partial correlation coefficient (*r*) was used to measure the strength of the association between infection intensity and antibody levels, controlling for the effect of age. To investigate whether the mean antibody levels or mean infection intensity differed significantly between preschool and primary school-aged children, independent *t* tests were used. The effect of sex, age group and village on the mean infection intensity and on the antibody levels was investigated using general linear regression models. To determine whether infection prevalence differed between the two age groups and that prevalence derived from parasitological data differed from that based on serological data, Chi-square (χ^2) tests were used.

Age-dependent prevalence model

Infection prevalence based on the binary response variables derived from parasitology and serology as a function of age, was estimated parametrically using the method of generalized linear regression modelling. Letting n be the sample size under investigation, a_i the age of the ith child ($i = 1, \ldots n$) and $q(a)$ the proportion of infection-negative children at age a in the study population. The prevalence, which is the probability of being infected at age a, is given by: $\pi(a) = 1 - q(a)$ and estimated using the binary response variable Y_i as follows: $\pi(a) = P(Y_i = 1 \mid a_i)$. The generalized linear model with a complementary log-log link was applied to take into account the binary nature of the response (Mathei *et al*. 2006) and expressed parametrically as follows:

$$\pi(a) = 1 - \exp(-\alpha a^{\beta})$$

where α is the intercept and β is the slope, i.e. the coefficient representing the effect of age on the probability of being infection positive.

P values less than 0·05 were considered statistically significant in this study.

RESULTS

Infection intensity and antibody levels

The observed overall mean *S. haematobium* infection intensity based on egg counts was 17·40 eggs/10 mL urine (S.D. = 71·20) and the overall mean antibody levels was 0·62 OD (S.D. = 0·34). The mean infection intensity and antibody levels were significantly higher for the primary-school aged children compared with that for preschool-aged children as shown in Table 1. A large variability in egg counts was observed as indicated by the large S.D. of the mean for both age groups in Table 1. Based on parasitology, 7·1% of all children participating in this study carried heavy infections ($\geqslant$ 50 eggs/10 mL urine), 30·4% had light infections (1–49 eggs/10 mL urine) and 62·6% had no infection burden (0 eggs/10 mL urine) according to the WHO classes of infection intensity (WHO, 2002). Among the preschool-aged children, 3·1% had heavy infections, 15·5% had light infections and 81·4% had no infection and in primary school-aged children, 8·2% had heavy infections, 34·6% had light infections and 57·2% had no infection.

Table 2. *F* and *P* values from general linear regression models to test for the difference in mean infection intensity (transformed using log10[egg count +1]) and IgM antibody response directed against schistosome egg antigens by sex and village, adjusting for the effect of age

	Infection intensity	Antibody level
Variable	*F* (*P* value)	*F* (*P* value)
Age (years)	13·92 (<0·001)	47·47 (<0·001)
Sex (F *vs* M)	2·11 (0·147)	1·51 (0·220)
Village (1 *vs* 2)	0·49 (0·483)	0·48 (0·491)

Both infection intensity ($r = 0{\cdot}18$; $P < 0{\cdot}001$) and antibody levels ($r = 0{\cdot}31$; $P < 0{\cdot}001$) increased significantly with age. In addition, a positive correlation between infection intensity and antibody levels was found ($r = 0{\cdot}23$; $P < 0{\cdot}001$). Infection intensity or antibody levels were not associated with sex and village of origin allowing for the effect of age (Table 2).

Infection prevalence: parasitology vs serology

The overall observed infection prevalence based on the two diagnostic techniques was as follows: parasitology, 37·4% (95% CI: 33·0–42·0%) and serology, 71·5% (95% CI: 67·2–75·7%) and these

Fig. 1. Infection prevalence derived using parasitological and serological diagnostic methods by age group. The indicated bars are the 95% confidence intervals of the observed prevalence and the *P* values test for the differences in prevalence between the diagnostic methods for each age group. White bars = prevalence based on parasitology and grey bars = prevalence based on serology.

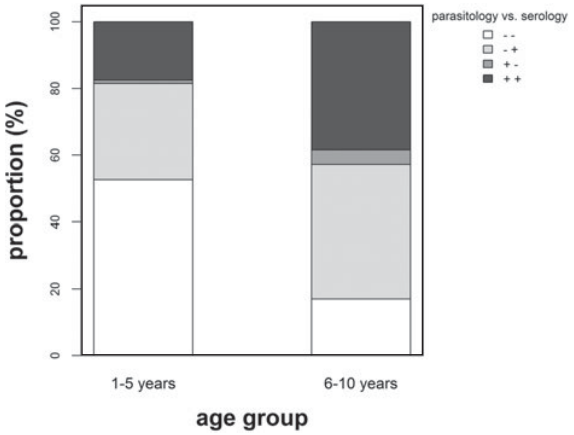

Fig. 2. Percentage proportion positive (+) *vs* negative (−) children diagnosed using parasitological and serological methods by age group. White stack: (− −) = negative for both diagnostic methods (1–5 years, n = 51; 6–10 years, $n = 58$), light grey stack (− +) = negative for parasitology but positive for serology (1–5 years, $n = 28$; 6–10 years, $n = 137$), grey stack (+ −) = positive for parasitology but negative for serology (1–5 years, $n = 1$; 6–10 years, $n = 15$) and dark grey stack (+ +) = positive for both diagnostic methods (1–5 years, $n = 17$; 6–10 years, $n = 131$).

differed significantly ($\chi^2 = 102{\cdot}12$; $P < 0{\cdot}001$). In addition, the infection prevalence based on serology was found to be significantly higher than the prevalence derived from parasitology for both age groups (Fig. 1). No significant difference in infection prevalence between male and female children was observed (parasitology, $\chi^2 = 0{\cdot}79$; $P = 0{\cdot}374$, and serology, $\chi^2 = 0{\cdot}15$; $P = 0{\cdot}703$).

The proportion of children classified as infection negative using the parasitological technique in preschool-aged children was significantly lower ($\chi^2 = 4{\cdot}11$; $P = 0{\cdot}043$) compared with that in primary school-aged children (Fig. 2). For this study, only 16 (3·7%) children (10 female and 6 male, age $\geqslant 5$ years) were found egg positive but classified as infection negative using the serological diagnostic method. In addition, for 9 of these children, eggs were detected only in one urine sample, with a mean count of 4 eggs/10 mL urine or less.

Age-dependent prevalence profiles: parasitology vs serology

The results for estimated regression coefficients and S.E. used to determine the age-dependent infection prevalence based on parasitological *vs* serological data were as follows: intercept, α: 0·04 (S.E. = 0·02) *vs* 0·12 (S.E. = 0·05) and slope, β: 1·23 (S.E. = 0·27) *vs* 1·25 (S.E. = 0·19). Infection prevalence increased with age in a similar pattern for both diagnostic methods, however the rate of increase for serology was higher compared with that of parasitology (Fig. 3). In addition, the infection prevalence derived using the serological technique was higher compared with the prevalence based on parasitological diagnostic method and this discrepancy increased with age (Fig. 3). The infection levels for primary school-aged children based on serology belonged to the high-risk WHO category (prevalence $\geqslant 50\%$) compared with the moderate-risk category implied by the parasitological diagnostic method.

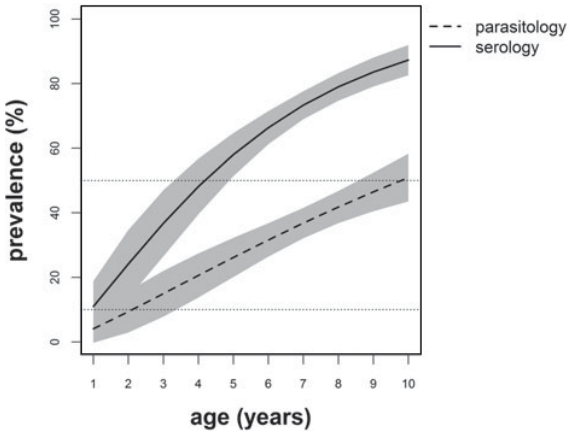

Fig. 3. Predicted age-related infection prevalence profiles derived from parasitological (dashed line) and serological (solid line) diagnostic methods. The grey shadings around the prevalence curves indicate the 95% confidence intervals. The horizontal dashed lines indicate the moderate (10%) and high (50%) infection-risk cut-offs for control regimens as defined by the World Health Organization (WHO, 2002).

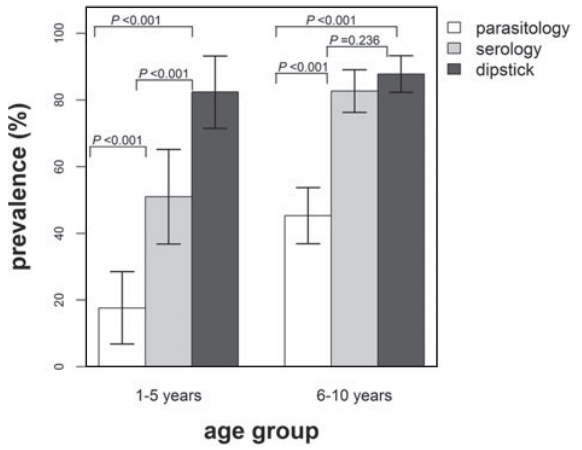

Fig. 4. Infection prevalence derived using parasitological, serological and dipstick microhaematuria diagnostic methods by age group for a subset of the study population ($n = 190$). The indicated bars are the 95% confidence intervals of the observed prevalence and the *P*-values test for the differences in prevalence between the diagnostic methods for each age group. White bars = prevalence based on parasitology, grey bars = prevalence based on serology and dark grey bars = prevalence based on dipstick microhaematuria.

Dipstick microhaematuria diagnostic method

Infection prevalence derived using the dipstick microhaematuria test was compared with the infection prevalence determined using the parasitological diagnostic method on 190 children (Fig. 4). The overall infection prevalence derived from the dipstick microhaematuria in this subset of the study population was 86·3% (95% CI: 81·9–91·7%) compared with 37·9% (95% CI: 30·9–44·9%) based on parasitology and 74·2% (95% CI: 67·9–80·5%) derived from the serological diagnostic method. Furthermore, infection prevalence based on dipstick microhaematuria was significantly higher compared with prevalence based on parasitology for both age groups. It was further noted that none of the egg-positive children were diagnosed as infection negative using dipstick microhaematuria and 4 (2·9%) primary school-aged children were found egg positive but with no microhaematuria detected in urine.

DISCUSSION

Following successful advocacy by the World Health Assembly (WHA, 2001), repeated MDA has become the key control strategy to combat schistosomiasis (WHO, 2002, 2013), with frequency of treatment dependent on the pre-determined infection prevalence (WHO, 2002). However, taking into consideration the reduced sensitivity of the parasitological diagnostic technique in children carrying light infections (Engels *et al*. 1997; Coulibaly *et al*. 2013), it is imperative that additional sensitive diagnostic tools are incorporated to improve the determination of infection levels. In this study we compared levels of *S. haematobium* infection obtained by the parasitological (egg count) method to the serological technique. In addition, these infection levels were compared between preschool and primary school-aged children to elucidate the need for inclusion of the neglected preschool age group into control programmes. The implications of infection levels determined in this study for the WHO recommended MDA regimens were also investigated.

In agreement with other studies using different diagnostic tools (Kahama *et al*. 1998; Kanamura *et al*. 2002; Lengeler *et al*. 2002; van Dam *et al*. 2004), infection levels (infection intensity and prevalence) increased significantly with age in this study. Unsurprisingly, infection intensity was positively correlated with anti-egg IgM antibody levels, since children accumulate infection with the associated increase in exposure to schistosome antigens (Stothard *et al*. 2011*b*). More importantly, the results of this study revealed significant infection prevalence in preschool-aged children, further concurring with findings from recent studies on the infection burden in this age group (Garba *et al*. 2010; Sousa-Figueiredo *et al*. 2010; Mutapi *et al*. 2011; Stothard *et al*. 2011*a*). These findings implicate a risk of preschool-aged children developing severe pathology due to chronic infection if left untreated (Stothard *et al*. 2011*b*; Ekpo *et al*. 2012). Hence the inclusion of these children in control programmes should be considered fundamental for improved and balanced childhood health (Garba *et al*. 2010).

This study revealed, in contrast with serology, that the parasitological technique approach underestimated infection prevalence in both age groups. These findings are indicative of reduced sensitivity of the parasitological technique since the majority of children in our study population carried light infection. In addition, following the WHO guidelines (WHO, 2002), infection prevalence derived from the serological method suggested a more frequent treatment intervention for this study population compared with that implicated by the parasitological technique. These findings further demonstrate that the use of different diagnostic techniques can be of importance in decision-making about suitable control strategies to implement. The WHO system is based upon parasitology, and was developed before the contribution of light infections (not detected via egg counts) to pathology was fully realized. The combination of additional diagnostics which can detect low infection levels and better definition of morbidity arising from low infections in *S. haematobium* infections (as recently summarized by King and Bertsch, 2013) support the current efforts for including preschool-aged children in schistosomiasis control programmes (Stothard *et al*. 2013).

The small proportion of schistosome egg-positive children in this study who were classified as infection negative using the serological technique can theoretically be attributable to two reasons: (1) contamination of the urine samples (Mutapi, 2011), which can occur as a result of instruments not being thoroughly cleaned or urine contamination with stool, especially for young female children; and (2) individual variability in mounting an immune response against the parasite antigens (Stothard *et al*. 2011*a*).

Similar patterns of age-dependent infection prevalence profiles were observed for both diagnostic methods, indicative of early exposure to infection and the accumulation of infection as children grow older (Garba *et al*. 2010; Stothard *et al*. 2011*a*). Overall, the estimated age-dependent prevalence based on serology was higher compared with that derived from parasitology, and this discrepancy between infection levels obtained from the two diagnostic methods also increased with age. Consequentially, the observed age-prevalence patterns indicated that the required intervention strategies varied with age.

The use of dipstick microhaematuria in this study detected higher prevalence of infection equally for both preschool and primary school-aged children in comparison to the parasitological method. These findings highlighted the usefulness of dipstick microhaematuria as an additional diagnostic tool in children carrying light infections, in agreement with findings from other recent studies (King and Bertsch, 2013). Haematuria due to glomerular causes has been reported in children (Meyers, 2004), thus caution should be exercised when interpreting the high prevalence of microhaematuria in preschool-aged children. Further studies are needed to elucidate levels of haematuria attributable to schistosome infection in this age group. French *et al*. (2007) recommended comparison of dipsticks sourced from different manufacturers to assess the effect of quality on the test results. In this study we used dipsticks sourced from two different companies (Uristix® from Plasmatec and Multistix® from Bayer) and they gave comparable results, supporting the robustness of our findings.

CONCLUSION

In conclusion, this study showed significant *S. haematobium* infection levels among untreated preschool and primary school-aged children who were life-long residents of an endemic area. Infection intensity and prevalence increased rapidly from early childhood, highlighting the need for treatment of the preschool-aged children. This study further highlighted the essential need for incorporating preschool-aged children into control programmes for the health benefits of treatment currently being offered to their older counterparts and thus prevent creating a childhood health inequity (Mutapi *et al*. 2011; Stothard *et al*. 2011*b*, 2013). Infection prevalence based on serology suggested a more frequent MDA regimen to that implied by the parasitological technique. We reiterate the importance of using sensitive diagnostic methods to improve accuracy in estimating true infection prevalence as this has implications on the required MDA regimen for the population. In our study, serology was highlighted as a valuable sensitive diagnostic tool that could be applied in conjunction with the parasitological technique. The findings of this study revealed that dipstick microhaematuria was equally sensitive in diagnosing infection in both preschool and primary-school aged children. Further evaluation of detection of microhaematuria using dipsticks as an additional diagnostic tool for *S. haematobium* infection in preschool-aged children is recommended.

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APPENDIX

Table A1. Description of the different sample sizes of the study population (total, $n = 438$) for parasitology and serology data by age group

		Serology		
Age group	Parasitology	Negative	Positive	Total
1–5 years	Negative	51	28	79
	Positive	1	17	18
6–10 years	Negative	58	137	195
	Positive	15	131	146
	Total	125	313	438

Table A2. Description of the different sample sizes of the subset (total, $n = 190$) of the study population for parasitology, serology and dipstick microhaematuria data by age group

		Serology		Dipstick microhaematuria	
Age group	Parasitology	Negative	Positive	Negative	Positive
1–5 years	Negative	25	17	9	33
	Positive	0	9	0	9
6–10 years	Negative	22	54	13	63
	Positive	2	61	4	59
	Total	49	141	26	164

PLOS | NEGLECTED TROPICAL DISEASES

RESEARCH ARTICLE

Identifying and Evaluating Field Indicators of Urogenital Schistosomiasis-Related Morbidity in Preschool-Aged Children

Welcome M. Wami[1,2]*, Norman Nausch[1,2¤a], Nicholas Midzi[3¤b], Reggis Gwisai[4], Takafira Mduluza[5], Mark Woolhouse[2], Francisca Mutapi[1,2]

1 Institute of Immunology and Infection Research, School of Biological Sciences, University of Edinburgh, Edinburgh, United Kingdom, **2** Centre for Immunity, Infection and Evolution, School of Biological Sciences, University of Edinburgh, Edinburgh, United Kingdom, **3** National Institute of Health Research, Causeway, Harare, Zimbabwe, **4** Ministry of Health and Child Care, Murewa District Hospital, Murewa, Zimbabwe, **5** Department of Biochemistry, University of Zimbabwe, Mount Pleasant, Harare, Zimbabwe

¤a Current address: Pediatric Pneumology and Infectious Diseases Group, Department of General Pediatrics, Neonatology and Pediatric Cardiology, University Children's Hospital, Duesseldorf, Germany
¤b Current address: Department of Medical Microbiology, College of Health Sciences, University of Zimbabwe, Harare, Zimbabwe
* W.M.Wami@sms.ed.ac.uk

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Data Availability Statement: We do not have ethical clearance from the Medical Research Council of Zimbabwe or the Research Council of Zimbabwe to make the raw data available. Other users would have to apply for permission to use the data from these bodies who granted us permission to conduct the study. The Medical Research Council of Zimbabwe is contactable is via an online email system: http://www.mrcz.org.zw/index.php?option=com_contact&view=contact&id=4&Itemid=10

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Abstract

Background

Several studies have been conducted quantifying the impact of schistosome infections on health and development in school-aged children. In contrast, relatively little is known about morbidity levels in preschool-aged children (≤5 years) who have been neglected in terms of schistosome research and control. The aim of this study was to compare the utility of available point-of-care (POC) morbidity diagnostic tools in preschool *versus* primary school-aged children (6–10 years) and determine markers which can be used in the field to identify and quantify *Schistosoma haematobium*-related morbidity.

Methods/Principal Findings

A comparative cross-sectional study was conducted to evaluate the performance of currently available POC morbidity diagnostic tools on Zimbabwean children aged 1–5 years (n=104) and 6–10 years (n=194). Morbidity was determined using the POC diagnostics questionnaire-based reporting of haematuria and dysuria, clinical examination, urinalysis by dipsticks, and urine albumin-to-creatinine ratio (UACR). Attributable fractions were used to quantify the proportion of morbidity attributable to S. haematobium infection. Based on results of attributable fractions, UACR was identified as the most reliable tool for detecting schistosome-related morbidity, followed by dipsticks, visual urine inspection, questionnaires, and lastly clinical examination. The results of urine dipstick attributes showed that proteinuria and microhaematuria accounted for most differences between schistosome egg-positive and negative children (T=-50.1; $p<0.001$). These observations were consistent in preschool vs. primary school-aged children.

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Conclusions/Significance

Preschool-aged children in endemic areas can be effectively screened for schistosome-related morbidity using the same currently available diagnostic tools applicable to older children. UACR for detecting albuminuria is recommended as the best choice for rapid assessment of morbidity attributed to *S. haematobium* infection in children in the field. The use of dipstick microhaematuria and proteinuria as additional indicators of schistosome-related morbidity would improve the estimation of disease burden in young children.

Author Summary

Schistosomiasis is a major parasitic disease affecting children in Africa, with impacts on health, growth and cognitive development. Recently, the World Health Organization has recommended inclusion of preschool-aged children ($\leq$5 years) in schistosome control programmes. However, so far the performance of available morbidity diagnostic tools has not been thoroughly evaluated in this age group. To address this knowledge gap, we conducted a study in preschool children comparing the utility of currently available point-of-care tools for diagnosing *Schistosoma haematobium*-related morbidity, namely: questionnaire-reported haematuria and dysuria, clinical examination, dipstick urinalysis, and measurement of urine albumin-to-creatinine ratio (UACR). We also investigated the performance of these tools in older children (6–10 years). Our study identified UACR as the most reliable tool for detecting schistosome-related morbidity in terms of the morbidity attributable to schistosome infection, followed by dipsticks, visual urine inspection, questionnaires, and lastly clinical examination The study further showed that the tools currently used in school-aged children for diagnosing schistosome-related morbidity can be extended to preschool children, allowing easier integration of this age group into treatment and monitoring programmes.

Introduction

Urogenital schistosomiasis is a major parasitic disease caused by *Schistosoma haematobium* affecting children in Africa, with negative impacts on child health, growth and cognitive development [1]. Chronic infection with the parasite can cause anaemia, malnutrition, and organ complications such as bladder fibrosis and kidney failure [2]. Schistosome control programmes focus on preventive chemotherapy with the antihelminthic drug of choice, praziquantel, to reduce or prevent the development of severe morbidity due to schistosome infection, and thereby improving health of the infected individuals and communities [3]. In order to achieve these goals and evaluate the effects of control programmes, an understanding of the morbidity due to schistosome infection is essential [4]. This requires the use of reliable rapid diagnostic tools that can be used in the field [5].

In recent years progress has been made towards improving methods for measuring *S. haematobium*-related morbidity and various techniques have been evaluated in older children and adult populations [5]. For example, ultrasonography has been shown to be effective in detecting organ-specific morbidity [6,7]. However, the need for specialized equipment and trained personnel reduces its utility for large population studies in the field. Urinalysis has been used as a rapid indirect assessment tool for early urinary tract morbidity due to schistosomiasis [8].

In addition, standardized questionnaires recommended by the WHO for rapid screening of *S. haematobium* infection and morbidity have been extensively used in endemic regions [9]. Most of these studies have focused on older children, typically primary school-aged children (6–10 years), or older individuals.

The WHO has recently recommended the inclusion of preschool children (aged 5 years and below) in schistosome control programmes [10], but the performance of the currently available point-of-care (POC) diagnostic tools for detecting schistosome-related morbidity have not yet been systematically evaluated in this age group. In addition, the utility of these POC tools has not been compared in a single study between preschool and primary school-aged (6–10 years) children, who are the current main targets of schistosome control programmes. Measuring the burden of schistosome disease in the whole population, including preschool children is important for the assessment of the effectiveness of control programmes and thus their prioritization and sustenance in affected countries (often with limited health budgets). Although extensive work has been done and a few recent studies published on morbidity due to *S. mansoni* infection in preschool children [11], to date there is still a paucity of studies quantifying the burden of *S. haematobium*-related morbidity in preschool children and the applicability of current POC morbidity diagnostics in these young children has not been extensively evaluated. To address this knowledge gap, we conducted a study in preschool and primary school children endemically exposed to *S. haematobium infection* assessing the utility of available diagnostic tools in identifying POC markers of schistosome-related morbidity.

The first aim of the study was to characterise the morbidity in the children detected using the available POC tools. Since the morbidity markers currently used are general as opposed to being schistosome specific, they may detect morbidity unrelated to schistosome infection. Therefore, the second aim of the study was to relate the measures of morbidity to schistosome infection and determine the fraction of morbidity attributable to schistosome infection. The overall results would allow us to determine if POC diagnostics available for use in primary school-aged children can be reliably used in the field to quantify and monitor levels of morbidity attributable to *S. haematobium* infection in young children aged 5 years and below.

Materials and Methods

Ethical statement

Ethical and institutional approval for the study was obtained from the Medical Research Council of Zimbabwe and the University of Zimbabwe, respectively. Permission to conduct the study was received from the Provincial Medical Director, the District Educational Officer, and Heads of schools in the study area. Study aims and procedures were explained to participants, and their parents/guardians in the local language, Shona. Prior to enrolment of study participants, written informed consent was obtained from parents/guardians and oral assent obtained from children. The children were recruited into the study on voluntary basis and were free to withdraw at any time with no further obligation. After sample collection, participants were offered treatment with the standard dose of 40 mg/kg praziquantel, administered by the local physician. The praziquantel drug was procured from a local supplier (Pharmaceutical and Chemical Distributors (Pvt) Ltd, Harare, Zimbabwe), registered and licensed to sell the drug in Zimbabwe.

Study area

The cross-sectional study was conducted in Murewa district, in the north-east of Zimbabwe (31°90'E; 17°63'S) where *S. haematobium* is endemic. Prevalence of *S. mansoni* was low (<10%) [9] in this current study population as previously reported in other studies conducted

in the same area [12,13]. There were no soil-transmitted helminths infections detected in this study population.

Participants

Children aged 1–10 years were recruited from crèches, early child development centres, and local primary schools between February 28, and March 09, 2012. To be included in this study, participants had to meet the following criteria: (1) been lifelong residents of the study area, (2) had no prior history of antihelminthic treatment (assessed by questionnaires administered to parents/guardians for all children), and (3) provided at least two urine, and two stool samples for parasitological examinations on consecutive days. The exclusion criteria were: (1) presenting with clinical symptoms of tuberculosis or malaria/fever, (2) recent major illness/operation, and (3) diagnosed positive for soil-transmitted helminths. None of the children were excluded based on these criteria.

Parasitology and serology

S. haematobium infection was determined by microscopic enumeration of eggs in urine processed using the standard urine filtration method [14]. Children were classified as infected if at least one parasite egg was detected in any of their urine samples collected on consecutive days. Infection intensity was defined as the arithmetic mean egg counts/10 mL of at least two urine samples collected on three consecutive days. Stool samples were processed using the Kato-Katz method, with duplicate thick smears (41.7 mg) performed per sample [15], and subsequent egg enumeration by microscopy for the diagnosis of *S. mansoni* and soil-transmitted helminths. Children were designated infected with *S. mansoni* or soil-transmitted helminths if at least one parasite egg was detected in any of the two stool samples collected on consecutive days. A small proportion, 6.0% (n = 18) of the children in our study was found positive for S. mansoni. We compared the morbidity characteristics of these children to those of a random sample drawn from age and sex matched S. mansoni negative children and no differences were observed, hence these children were kept in our study for the final analyses. None of the children in this study were found positive for STHs.

We have recently shown that egg count lacks sensitivity in diagnosing light schistosome infections in children [16]. Thus, in addition to parasitology, IgM antibody responses directed against soluble egg antigens (SEA) were used to improve the diagnosis of *S. haematobium* infection. Details of the protocols used to quantify the serum antibody levels are published elsewhere [17]. Children were categorized as infected based on serology if their anti-egg IgM antibody levels were more than two standard deviations above the mean estimated from sera of negative controls, as outlined in our recently published study [16].

Morbidity measurement

Urinalysis. Urine samples collected on the first day of the survey were examined for visible haematuria. Uristix reagent strips (Uripath, Plasmatec, UK) were used to test for the presence of nitrites, leucocytes, blood (microhaematuria), proteins (proteinuria), and physical characteristics (pH, specific gravity). To assess observer bias in dipstick readings, a random sample of 102 of the 298 urine samples was further tested using the Multistix 10SG (Bayer, UK), and the results read automatically using Siemens' CLINITEK Status+ Analyzer (Bayer, UK). For all the attributes tested, a high proportion of overall agreement ($p_{overall}$>60.0%) between the two dipstick tests was noted, showing no evidence of significant observer effect. CLINITEK Microalbumin Reagent Strips (Bayer, UK) were used to determine urine albumin-to-creatinine ratio (UACR) threshold levels on first day urine samples. Following manufacturer's guidelines, high-abnormal

UACR (>33.9 mg/mmol) was used to ascertain presence of albuminuria [18], a biological marker of urinary tract infection and an early predictor of progressive kidney disease [8].

Questionnaires. A pre-tested questionnaire on recent/current presence of haematuria and dysuria, constructed in English and translated to the local language, Shona, was administered to parents/guardians of preschool-aged children. An alternative version of the questionnaire was administered to the primary school-aged children.

Clinical examination. All participants underwent a non-intrusive clinical examination, involving abdominal palpation, conducted by experienced study clinicians to determine current health status and schistosome-related anomalies (e.g., epigastric or abdominal pains).

Statistical methods

Sample size calculation. Our pre-study simulations revealed that a sample size of 129 children would provide 80.0% power to detect age group related differences in infection prevalence differences at $\alpha = 0.05$, allowing for 5.0% non-compliance loss. These sample size calculations were based on the expected overall *S. haematobium* infection prevalence of 40.0% (for 1–5 years) and 60.0% (for 6–10 years), with information obtained from preliminary studies conducted in the same study area. Our final sample sizes for variables of interest were sufficiently large for statistical analyses (Fig. 1).

Statistical analyses. Correlations between continuous variables were measured using the Pearson's correlation coefficient (r). The phi-coefficient (φ) was used for dichotomous variables. The chi-square (χ^2) test was used to determine associations between different markers of morbidity by sex, age-group or *S. haematobium* infection status. Multiple logistic regressions were used to investigate factors influencing the prevalence of schistosome-related morbidity. Each of the morbidity indicators was included as a response variable, with sex (male *vs*. female), age-group (1–5 *vs*. 6–10 years) and *S. haematobium* infection status (determined by parasitology or serology) or infection intensity ($\log_{10}$[egg count+1]-transformed) included as risk factors in the models. Two-way interaction effects were included in model building, however, none were found to be significant and hence were subsequently dropped from the final models used for inferences.

Non-metric multidimensional scaling (NMDS) was used to explore the variability in dipstick attributes between children. For an outline of the NMDS modelling steps (see S1 File Supporting Information which explains the algorithm steps followed in this study and the test statistics used to evaluate the NMDS models). Correlation coefficients were used to identify dipstick attributes contributing most to overall variability in schistosome-related morbidity. The proportion of variability explained by each of the NMDS axes was measured using the coefficient of determination (R^2R^2). The multi-response permutation procedure (MRPP) was used to test the null hypothesis of no significant differences between subgroups. For each pairwise comparison, the resultant test-statistic (T) was reported along with the corresponding p-value [19].

The risk of morbidity for each age group was estimated using prevalence ratios, where a prevalence ratio greater than one indicated a positive association between schistosome infection and presence of related morbidity. The Breslow-Day test with Tarone's adjustment for small sample sizes [20], was used to assess whether the probability of detecting morbidity using the different diagnostic tools in infected children differed between 1–5 years and 6–10 years old children. The population attributable fraction, and attributable fraction infected were used to estimate the proportion of morbidity in the whole study population and among infected children that could be attributed to *S. haematobium* infection respectively, adjusting for the effects of sex and age group. Furthermore, these estimates were used to compare the utility of the different diagnostic tools for detecting schistosome-related morbidity. Approximate 95% confidence intervals were calculated using the method described elsewhere [21]. For meaningful

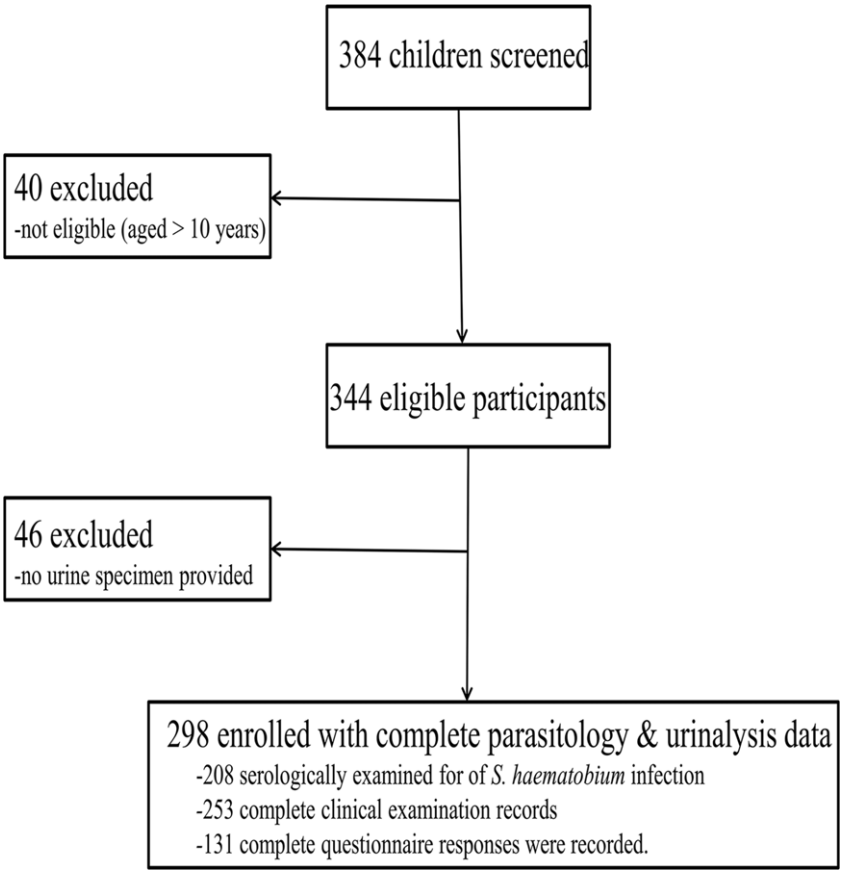

Fig 1. Flowchart indicating number of children enrolled in the study and excluded from the final analysis.

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interpretations, attributable fractions were only estimated for the morbidity markers with a prevalence ratio (PR) significantly greater than one.

Sample size calculations were performed using StatXact v.8 (Cystel Software Corp, Cambridge, MA, USA). The NMDS analysis was performed using PCORD 6.08 (MjM Software, Gleneden Beach, Oregon, USA). Statistical modelling and tests for associations were performed using SAS 9.3 (SAS Institute Inc., Cary, NC, USA). In all analyses, the level of significance was set at $p<0.05$.

Results

Demographics

298 children (1–5 years: n = 104, median = 4 years; 6–10 years: n = 194, median = 8 years) fulfilled the study criteria (Fig. 1), and these comprised of 142 (47.7%) males, and 156 (52.3%) females.

Schistosome infection levels

The overall prevalence of *S. haematobium* infection determined by parasitological examination was 35.9% (95% CI: 30.4–41.4%). When looking at infection intensities, 28.9% (95% CI: 23.7–34.0%) and 7.0% (95% CI: 4.1–10.0%) of these children carried respectively light and heavy infection intensities according to the WHO categorizations [8]. Infection prevalence amongst primary children aged 6–10 years was 47.9% (95% CI: 40.8–55.0%), and was significantly higher ($\chi^2 = 35.0$; $p<0.001$) compared to infection prevalence of 13.5% (95% CI: 6.8–20.1%) observed in 1–5 years old children. However, there was no significant difference ($\chi^2 = 0.5$; $p = 0.466$) in the prevalence of infection between male and female children. Infection intensity increased significantly with age ($r = 0.4$; $p<0.001$), with the highest levels observed between the ages of 8–10 years. The prevalence of *S. haematobium* infection determined by serology was higher than that determined by egg counts in both age groups, 1–5 years: 52.9% (95% CI: 38.8–67.1%), and 6–10 years: 84.1% (95% CI: 78.3–89.9%).

Urinary dipstick morbidity markers

Dipstick-detected microhaematuria and proteinuria, contributed most to the observed variability in morbidity among children (taking into account urine's physical characteristics, pH and specific gravity), as indicated by the strong correlations (see S1 Table). The variability of morbidity differed significantly between *S. haematobium* egg negative and positive children ($T = -50.7$; $p<0.001$) and between the two age groups ($T = -19.3$; $p<0.001$), however there were no differences by sex ($T = -1.5$; $p = 0.089$). Furthermore, the observed differences were evident from the large NMDS ordination output distances between the respective subgroup centres shown in Fig. 2. Based on the serological diagnosis of infection, significant differences were also observed by infection status ($T = -14.0$; $p<0.001$), age group ($T = -6.5$; $p<0.001$), but not by sex ($T = -2.5$; $p = 0.068$). In addition, microhaematuria and proteinuria alone explained about two-thirds of the overall variability due to differences between infected and uninfected children (detected by either parasitology or serology).

Observed morbidity prevalence

The prevalence of dipstick microhaematuria was higher than questionnaire-reported haematuria, which in turn was also higher than visible haematuria (Fig. 3). The morbidity prevalence results illustrated in Fig. 3 revealed that children aged 6–10 years tended to report morbidity more frequently compared to parents/guardians of 1–5 years old children. In addition, albuminuria (detected by UACR) and dipstick proteinuria were observed in both age groups as shown in Fig. 3. A positive association of albuminuria with microhaematuria ($\varphi = 0.2$, $p = 0.002$), or proteinuria ($\varphi = 0.4$; $p<0.001$) was observed. In comparison to other diagnostic techniques investigated in this study, visual urine inspection, and clinical examination detected the least number of morbidity cases (Fig. 3).

Schistosome infection versus morbidity prevalence

Results of multiple logistic regression analyses revealed a significant positive association (in order of strength of the association) of visible haematuria, albuminuria, microhaematuria, and proteinuria with *S. haematobium* infection detected by parasitology as shown in Table 1. In addition, *S. haematobium* infection prevalence determined by serology was also found to be significantly associated with albuminuria and proteinuria, adjusting for the effects of sex and age group (Table 1). Significant increases in prevalence of albuminuria (Odds ratio (OR) = 5.5; $p<0.001$), visible haematuria (OR = 4.7; $p<0.001$), microhaematuria (OR = 3.4; $p = 0.005$),

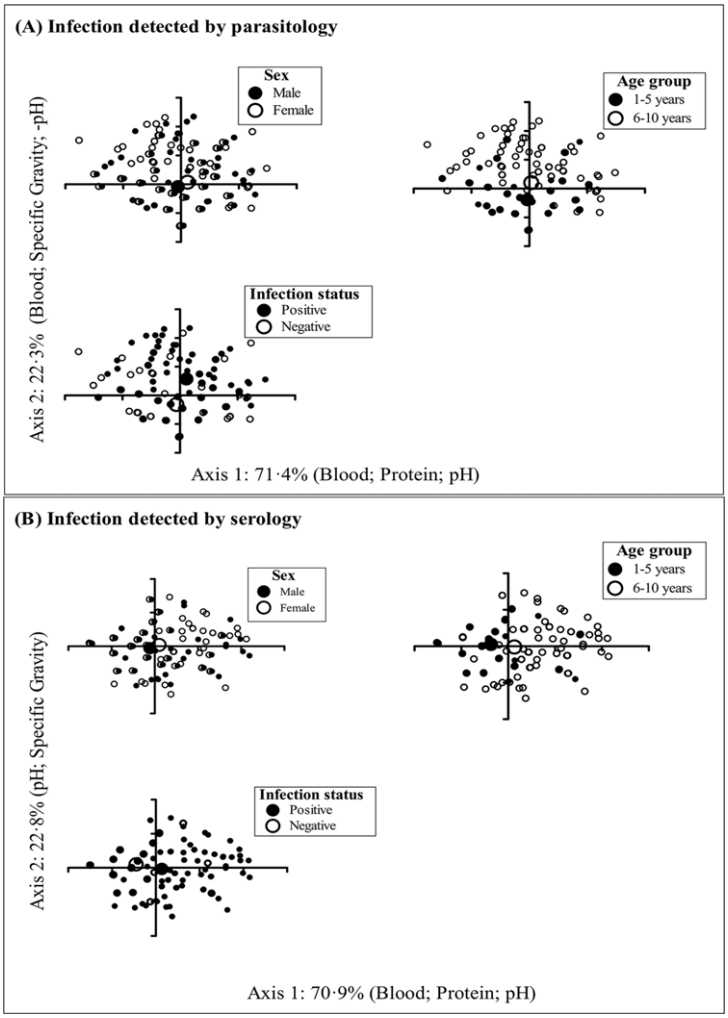

Fig 2. Non-metric multidimensional scaling (NMDS) ordination in 2-dimensional configurations by sex, age-group and *S. haematobium* infection status determined using parasitological (A) and serological diagnostic techniques (B). Subgroup centres are represented by the bigger closed (●), or open (○) points, and the distance between these centres is proportional to the level of dissimilarities between subgroups.

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and proteinuria (OR = 3.3; $p<0.001$) with infection intensity were observed. However, no significant associations between infection intensity and questionnaire-reported haematuria and dysuria, or clinical examination detected morbidity were found.

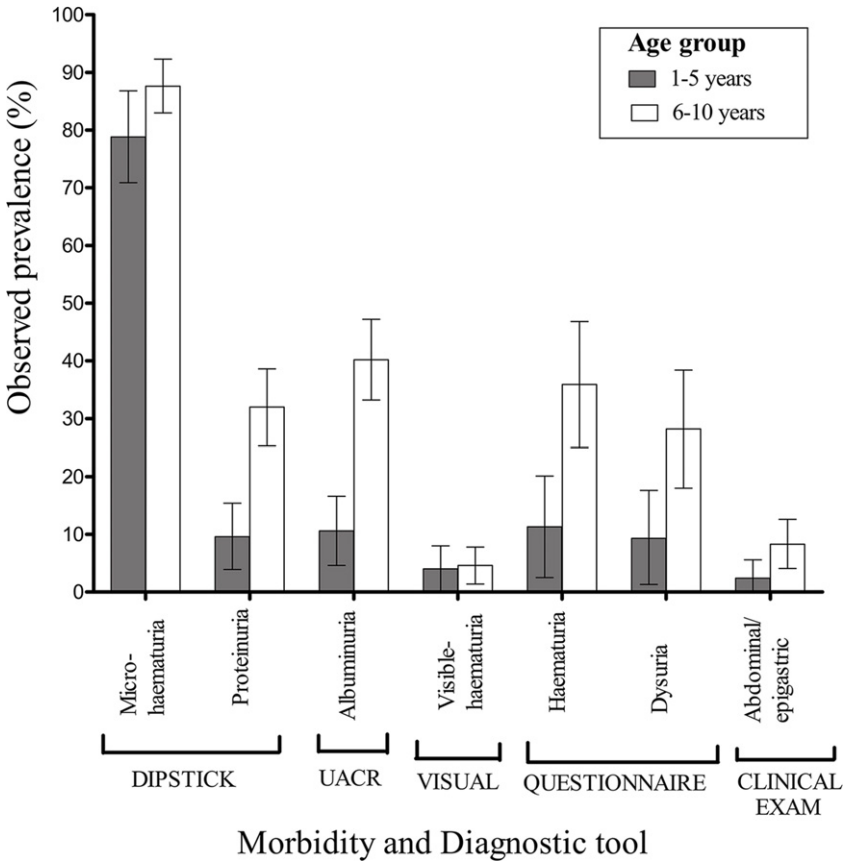

Fig 3. Observed prevalences of morbidity by age group, assessed using different diagnostic tools. Error bars indicate the 95% confidence intervals.

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Morbidity attributable to *S. haematobium* infection

Since the morbidity markers are not specific to schistosomes but are general markers of different physiological and biochemical processes, we went further to determine how much of the morbidity was attributable to schistosome infection. There was no significant difference in the estimated probability of detecting morbidity between 1–5 years and 6–10 years old children using each of the diagnostic tools (Table 2). In addition, from Table 2, it was observed that morbidity detected by dipsticks (microhaematuria and proteinuria), UACR (albuminuria), and urine inspection (visible haematuria) had prevalence ratios significantly greater than one. Clinical examination detected morbidity had the lowest prevalence ratio (Table 2). Furthermore, the results indicated that albuminuria was the dominant marker of schistosome attributable morbidity at population level, as well as amongst infected children (Fig. 4). Proteinuria and visible haematuria were also found to be highly attributable to schistosome infection among

Table 1. Multiple logistic regression odds ratios (OR) to investigate factors associated with the prevalence of morbidity assessed using different diagnostic tools.

			Infection detected by parasitology		Infection detected by serology	
Type of morbidity	**Diagnostic tool used**	**Variable**	**OR (95% CI)**	**p**	**OR (95% CI)**	**p**
Microhaematuria	Dipstick	Sex (M *vs*. F)	1.8 (0.9–3.4)	0.089	**2.7 (1.1–6.7)**	**0.031**
		Age group (1–5 *vs*. 6–10 years)	1.2 (0.6–2.4)	0.563	1.4 (0.5–3.9)	0.472
		Infection status (negative *vs*. positive)	**3.4 (1.5–7.9)**	**0.005**	0.9 (0.3–2.7)	0.902
Proteinuria	Dipstick	Sex (M *vs*. F)	1.2 (0.6–2.1)	0.594	1.2 (0.6–2.3)	0.564
		Age group (1–5 *vs*. 6–10 years)	**2.5 (1.2–5.5)**	**0.019**	2.0 (0.8–4.9)	0.145
		Infection status (negative *vs*. positive)	**3.3 (2.2–5.0)**	**<0.0001**	**4.5 (1.5–13.6)**	**0.007**
Albuminuria	UACR	Sex (M *vs*. F)	0.8 (0.4–1.5)	0.528	0.8 (0.4–1.5)	0.481
		Age group (1–5 *vs*. 6–10 years)	**3.1 (1.5–6.7)**	**0.004**	**3.4 (1.3–8.5)**	**0.011**
		Infection status (negative *vs*. positive)	**5.5 (3.4–8.9)**	**<0.0001**	**33.9 (4.5–254.0)**	**0.001**
Haematuria[a]	Visual inspection	Sex (M *vs*. F)	1.1 (0.3–3.6)	0.876	1.4 (0.3–5.9)	0.690
		Age group (1–5 *vs*. 6–10 years)	0.5 (0.1–1.9)	0.299	1.0 (0.2–5.3)	0.991
		Infection status (negative *vs*. positive)	**7.8 (1.8–34.4)**	**0.007**	-	-
Haematuria	Questionnaire	Sex (M *vs*. F)	1.0 (0.5–2.4)	0.931	1.6 (0.6–4.2)	0.349
		Age group (1–5 *vs*. 6–10 years)	**3.9 (1.4–10.8)**	**0.009**	**5.5 (1.1–27.6)**	**0.037**
		Infection status (negative *vs*. positive)	1.4 (0.6–3.3)	0.443	2.1 (0.4–11.2)	0.385
Dysuria	Questionnaire	Sex (M *vs*. F)	0.6 (0.3–1.6)	0.325	0.6 (0.2–1.4)	0.223
		Age group (1–5 *vs*. 6–10 years)	**4.1 (1.3–12.6)**	**0.013**	2.5 (0.7–9.1)	0.168
		Infection status (negative *vs*. positive)	1.0 (0.4–2.4)	0.926	1.6 (0.4–6.9)	0.531
Abdominal/	Clinical exam	Sex (M *vs*. F)	0.9 (0.3–2.5)	0.826	1.2 (0.4–3.5)	0.788
epigastric[b]		Age group (1–5 *vs*. 6–10 years)	-	-	-	-
		Infection status (negative *vs*. positive)	0.9 (0.3–2.6)	0.882	1.2 (0.3–4.4)	0.821

Significant effects ($p<0.05$) are shown in bold.

[a]OR not adjusted for serological infection status;

[b]OR not adjusted for age group effect.

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Table 2. Estimates of prevalence ratios (PR) weighted by age group for each of the morbidity markers assessed using different diagnostic tools and test for homogeneity of the probability of detecting morbidity in infected children.

		Infection detected by parasitology			Infection detected by serology		
Type of morbidity	**Diagnostic tool used**	**PR (95% CI)**	**χ^2-statistic**	**p**	**PR (95% CI)**	**χ^2-statistic**	**p**
Microhaematuria	Dipstick	**3.3 (1.4–7.9)**	1.4	0.231	0.9 (0.5–2.6)	0.4	0.509
Proteinuria	Dipstick	**1.5 (1.2–1.8)**	0.2	0.666	**1.3 (1.1–1.5)**	2.5	0.114
Albuminuria	UACR	**2.4 (1.9–3.1)**	0.01	0.927	**1.7 (1.4–1.9)**	0.3	0.571
Haematuria	Visual inspection	**1.1 (1.0–1.2)**	0.002	0.989	**1.5 (1.3–1.7)**	[a]	[a]
Haematuria	Questionnaire	1.1 (0.8–1.5)	5.1	0.024	1.1 (0.9–1.5)	1.5	0.225
Dysuria	Questionnaire	1.0 (0.8–1.3)	3.1	0.078	1.1 (0.8–1.6)	5.5	0.017
Abdominal/epigastric	Clinical exam	1.0 (0.3–2.9)	3.0	0.081	1.0 (0.9–1.1)	[a]	[a]

Comparisons between preschool-aged (1–5 years) versus primary school-aged (6–10 years) children. Prevalence ratios significantly higher than 1 are shown in bold.

[a]Test statistic could not be computed.

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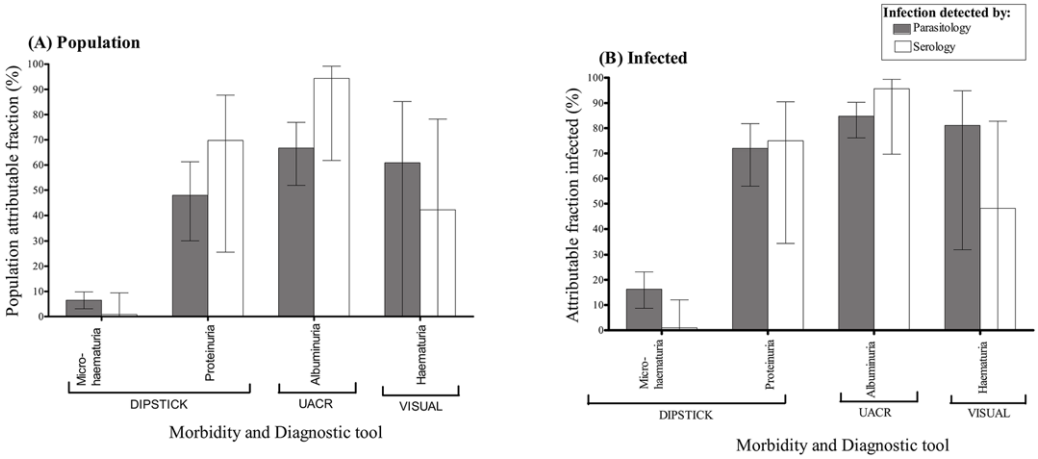

Fig 4. Estimated proportion of morbidity attributable to *S. haematobium* infection. (A) Population attributable fraction, (B) Attributable fraction infected.

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infected children. Although a high crude prevalence of microhaematuria was observed initially, the analyses revealed that a relatively small proportion of microhaematuria was attributed to *S. haematobium* infection (Fig. 4). The attributable fractions among infected children estimated by age group strata (see S1 Fig) showed a similar trend to the overall estimated attributable fractions noted above.

Discussion

Until recently, most schistosome control programmes in Africa aimed at reducing development of severe morbidity and improving child health have focused on regular school-based deworming strategies, targeting children above five years old [22–24]. By focusing treatment upon the school-aged population, children of preschool-age have been previously neglected in terms of research and control [25]. Consequently, less is known about the levels of schistosome-related morbidity in this age-group. Furthermore, research studies evaluating the performance of the current POC markers of schistosome-related morbidity in children aged five years and below are still limited [11]. Estimation of disease burden due to schistosome infections in children has been further complicated by the fact that signs and symptoms commonly associated with schistosomiasis can also be due to other causes [26]. In the absence of a gold standard POC morbidity diagnostic technique, several methods have been used in studies from different endemic settings in older children (≥6 years) and adult populations [5]. Our study focused on the tools used in the field; the WHO approved questionnaire-based reporting of haematuria and dysuria, clinical examination by qualified clinicians, routinely used dipstick tests measuring several urine attributes, and UACR (for detecting albuminuria) which has previously been evaluated for schistosome morbidity detection [8]. We investigated how these tools performed in preschool-aged children (1–5 years) compared to primary school-aged children (6–10 years), who are the current targets of schistosome control programmes.

Our study revealed that children of the two age groups carried quantifiable levels of morbidity as determined by these different diagnostic tools. This finding is in accordance with a recent

epidemiological study by Sacko and colleagues [27] who reported significant prevalence of urinary pathology in endemically exposed children. Of the several urine attributes tested using dipsticks, microhaematuria and proteinuria were significantly associated with *S. haematobium* infection, as it has been previously reported in several other studies [28–30]. A high proportion of children aged 5 years and below presented with microhaematuria in this study. More interestingly, the current study demonstrated that the performance of each of the different POC diagnostic tools for detecting morbidity did not differ between preschool and primary school-aged children infected with *S. haematobium*. These findings are important for planning of future interventions as they provide evidence that children $\leq$5 years can be effectively screened for praziquantel treatment using the available POC diagnostic tools applicable to older children and adult populations in the field [27,31].

Since the physical and biological features determined by these diagnostics can arise due to several conditions [32,33], we determined how much of the proportion of morbidity was attributed to *S. haematobium* infection. Based on the results of prevalence ratios and attributable fractions, UACR was identified as the most reliable tool for detecting schistosome-related morbidity, followed by dipsticks, visual urine inspection, questionnaires and lastly clinical examination. In addition, prevalence of albuminuria determined using UACR was positively associated with presence of microhaematuria and proteinuria detected by dipsticks. This finding suggests that these indicators used in combination can be a better predictor of the presence of urinary tract morbidity due to *S. haematobium* infection in children than using one test parameter alone, and thereby facilitating effective and timely interventions. The utility of albuminuria as a valuable indicator of schistosome-related morbidity in our study corroborates earlier findings in school-aged children by Sousa-Figueiredo and colleagues [34].

Although the proportion of children with visible haematuria was low in this study, it was noted that *S. haematobium* egg-positive children were eight times more likely to present with visible haematuria compared to egg-negative children. In addition, all children with visible haematuria were positive for *S. haematobium* infection detected using the serological diagnostic test. The majority of children in this study carried light infections, and this could explain the observed low prevalence of visible haematuria [35].

Since *S. haematobium* infection in endemic areas can easily be inferred from presence of blood in urine, questionnaire responses about recent/current presence of haematuria and dysuria can be used to assess schistosome-related morbidity. Our study showed some level of bias in the reporting of haematuria and dysuria between preschool-aged children, where the answers were provided by the parents/guardians and primary school-aged children, who responded to the questions themselves. One theoretical explanation for these observations could be that children easily mistook concentrated urine as blood in urine, but less likely so by adults and hence resulting in the overestimation of the prevalence of reported morbidity amongst the 6–10 years old children. These results therefore need to be interpreted with caution.

Physical clinical markers of morbidity were least attributable to schistosome infection, as previously mentioned. Our findings are consistent with a recent study by Agnew-Blais and colleagues [36], who also reported inadequacy of the physical examination method for assessing schistosome-related pathology in school-aged populations.

Nevertheless, there are some limitations when interpreting the results of our study. Firstly, given that approximately 30% of our study participants were not characterized for *S. haematobium* infection using the more sensitive serological diagnostic technique, caution must be applied when extrapolating the study findings. Secondly, in our stratified analysis the sample size was too limited to give precise estimates of schistosome-related morbidity prevalence measured using different markers; these results should be interpreted with caution.

Conclusions

Our results confirm that schistosome infection in preschool children does result in significant morbidity. These findings are in agreement with recent studies on *S. mansoni* in Uganda [11] and *S. haematobium* in Malawi [37], reiterating the need for anthelminthic treatment in preschool children. This study has gone further to identify morbidity diagnostics with large fractions attributable to schistosome infection, highlighting detection of albuminuria as the best choice for rapid assessment of morbidity attributed to *S. haematobium* infection in children in the field. Finally the study showed that in *S. haematobium* endemic areas, preschool-aged children can be effectively screened for schistosome-related morbidity using the same diagnostic tools applicable to primary school-aged children and adult populations These findings are of clinical and public health importance, as these tools can be used to identify affected individuals or subgroups, thereby facilitating focused and timely delivery of treatment, as well as evaluate the effectiveness of interventions for improved control.

Supporting Information

S1 Table. Table that displays the Non-metric multidimensional scaling (NMDS) correlations (r) between urinary dipstick attributes and the two ordination axes.
(DOC)

S1 Fig. Figure that illustrates the proportion of morbidity attributable to *S. haematobium* infection detected by parasitology, estimated by age group strata.
(TIF)

S1 File. Text that explains the NMDS modelling steps used in the study.
(DOC)

S2 File. STROBE Checklist.
(DOC)

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Author Contributions

Conceived and designed the experiments: FM NM TM. Performed the experiments: WMW NN NM RG TM FM. Analyzed the data: WW MW FM. Wrote the paper: WMW NN NM RG TM MW FM.

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