IMMUNE CORRELATES OF PROTECTION AGAINST RE-INFECTION OF SCHISTOSOMIASIS MANSONI IN SCHOOL CHILDREN BEFORE AND AFTER MASS DRUG ADMINISTRATION

By NDOMBI ERIC MAKUTO (M.Sc.,Immunology)

A thesis submitted in fulfilment of the requirements for the award of the degree of Doctor of Philosophy in Immunologyof the University of Nairobi

OCTOBER, 2015

DECLARATION

I, do hereby declare that this thesis is my original work and has not been presented for a degree or any other award in any other University

Signature..Date...

Eric Makuto Ndombi, Reg. No. I80/9050/2013

This thesis has been submitted for examination with our approval as university supervisors

1. Signature...Date..

   Prof Horace Ochanda

   School of Biological Sciences

   University of Nairobi

2. Signature...Date..

   Dr Diana Karanja

   Kenya Medical Research Institute

   Centre for Global Health Research, Kisumu

3. Signature………………………………..Date..

   Prof Dan Colley

   Centre for Tropical and Emerging Global Diseases

   University of Georgia, Athens, USA

DEDICATION

This work is dedicated to the people I love most, and owe a great deal for the immense sacrifices they have given for its accomplishment; my loving wife Eneddy Nelima, my son Wesley Ndombi and daughter Sarah Kayla 'Mama'. God bless you mightily.

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TABLE OF CONTENTS

DECLARATION ii

DEDICATION iii

ACKNOWLEDGEMENTS iv

TABLE OF CONTENTS vi

LIST OF FIGURES x

LIST OF TABLES xi

ACRONYMS AND ABBREVIATIONS xii

ABSTRACT xiv

CHAPTER ONE : INTRODUCTION 1

1.1 Background 1

1.1.1 Life cycle of schistosomes 2

1.2 Schistosomiasis distribution 3

1.2.1 Global schistosomiasis distribution 3

1.2.2 Schistosomiasis in Kenya 5

1.3 Economic importance of schistosomiasis 6

1.4 Schistosomiasis disease pathogenesis 8

1.4.1 Acute disease 8

1.4.2 Chronic disease 8

1.5 Diagnosis of schistosomiasis 9

1.6 Control of schistosomiasis 11

1.6.1 Chemotherapy using Praziquantel 11

1.6.2 Other drugs for treatment of schistosomiasis 12

1.6.3 Other control strategies 12

CHAPTER TWO : LITERATURE REVIEW 14

2.1.1 Schistosomiasis infection in children 14

2.2 Immune responses during acute and chronic stages 14

2.3 Morbidity in schistosomiasis mansoni 17

2.4 Schistosomiasis control using mass drug administration 19

2.5 Immune correlates of protection 22

2.6 Problem statement 23

2.7 Expected application of results 24

2.8 Research questions 24
2.9 Null hypotheses 25
2.10 Objectives 25
2.11 General objective 25
2.11.1 Specific objectives 25
CHAPTER THREE : GENERAL MATERIALS AND METHODS 26
3.1 Study area and subjects 26
3.2 Study design 27
3.2.1 Inclusion criteria 27
3.2.2 Exclusion criteria 28
3.2.3 Sample size 28
3.3 Specimen collection and handling 29
3.4 Laboratory procedures 29
3.4.1 Parasitology 29
3.4.2 Whole blood culture 29
3.4.3 Cell surface staining and flow cytometry 30
3.4.4 Cytokine ELISA 32
3.4.5 Serologic assays for schistosome-specific total IgG, IgE and IgG4 antibodies 33
3.4.6 Data processing and analysis 33
3.5 Ethical considerations 33
3.5.1 Informed consent process 34
CHAPTER FOUR : PRE-TREATMENT ANTI-SCHISTOSOME IMMUNOLOGICAL PROFILE IN A CROSS SECTION OF SCHOOL CHILDREN 35
4.1 Introduction 35
4.2 MATERIALS AND METHODS 38
4.2.1 Study area and subjects 38
4.2.2 Ethics statement 38
4.2.3 Immunological assays 39
4.2.3.1 Whole blood phenotyping 39
4.2.3.2Whole blood cultures 39
4.2.3.3 Antibody evaluation 39
4.2.3.4 Cytokine assays 40

4.3 RESULTS....41
4.3.1 Baseline characteristics....41
4.3.2 Pre-treatment anti-schistosome antibody levels....43
4.3.2.1 Anti-SEA and anti-SWAP antibody levels....43
4.3.2.2 Anti-TAL antibody levels....45
4.3.2.3 CD23+ B cell proportions....47
4.3.2.4 Cellular immune responses....48
4.4 DISCUSSION....51
CHAPTER FIVE : EFFECT OF TREATMENT WITH PRAZIQUANTEL ON SCHISTOSOME-SPECIFIC IMMUNE RESPONSES IN A GROUP OF SCHOOL-AGE CHILDREN....54
5.1 Introduction....54
5.2 MATERIALS AND METHODS....56
5.2.1 Study area and subjects....56
5.2.2 Study design....57
5.2.2.1 Inclusion criteria....57
5.2.2.2 Exclusion criteria....57
5.3 Specimen collection and handling....58
5.4 Laboratory procedures....58
5.4.2 Whole blood culture....58
5.4.3 Cell surface staining and flow cytometry....58
5.4.4 Cytokine ELISA assay....59
5.4.5 Serologic assays for schistosome-specific antibodies of given isotypes....60
5.4.6 Data processing and analysis....60
5.5 Ethical considerations....60
5.6. RESULTS....61
5.6.1 General characteristics....61
5.6.2 Anti-SEA and –SWAP antibody levels....64
5.6.3 Anti-SmTAL antibody responses....67
5.6.5 SEA- and –SWAP specific cytokine levels....72
5.7 Discussion....75
CHAPTER SIX : GENERAL DISCUSSION CONCLUSIONS AND RECOMMENDATIONS....81
6.1 Discussion....81

6.2 Conclusions 84
6.3 Recommendations 84
REFERENCES.. 86
Appendix I: Parent or Guardian Consent Form.. 98
Appendix II: Laboratory protocols 106
Appendix III: KEMRI ERC Study Approval 115

LIST OF FIGURES

Figure 1:1: Life cycle of *S. mansoni*, *S. japonicum*, and *S. haematobium*. 4

Figure 1:2: Global distribution of schistosomiasis 5

Figure 1:3: Prevalence of schistosomiasis in Kenya 6

Figure 1:4: *Schistosoma mansoni* egg...10

Figure 3.1: Map showing location of Asembo area……………………………..26

Figure 3.2: Flow cytometry data analysis density graphs………………………31

Figure 4.1: Plasma anti-soluble egg antigen (SEA) and anti-soluble worm antigen preparation (SWAP) antibody levels ……………………………..…………44

Figure 4.2: Plasma anti-SmTAL-1, SmTAL-2 and SmTAL-5 antibody levels...46

Figure 4.3: Percentage of CD19+ CD23+ B Cells………………………………47

Figure 4.4: Plasma supernatant interleukin (IL)-5 and IL-13 from whole blood cultured with SEA or SWAP……………..…………………………….………49

Figure 4.5: Plasma supernatant Interleukin (IL)-13 and Interferon (IFN)-γ from whole blood cultured with SEA or SWAP………….…………………………50

Figure 5.1: Plasma anti-soluble egg antigen (SEA) IgE, IgG and IgG4 antibody……………………………………………………………………...65

Figure 5.2: Plasma anti-soluble worm antigen prepration (SWAP) immunoglobulin (Ig)E, IgG and IgG4 antibody levels……………………….…66

Figure 5.3: Plasma anti-*Schistosoma mansoni* tegument allergen-like (SmTAL)-1, SmTAL-2 and SmTAL-5 immunoglobulin (Ig)E antibody levels……………68

Figure 5.4: Plasma anti-*Schistosoma mansoni* tegument allergen-like (SmTAL)-1, SmTAL-2 and SmTAL-5 immunoglobulin (Ig)G4 antibody levels…………69

Figure 5.5: Percentage of CD19+ CD23+ B cells……………….……………………70

Figure 5.6: Plasma supernatant IL-5, IL-10, IL-13 and IFN-γ from whole blood cultured with SEA or SWAP……………………………………………………72

Figure 5.7: Plasma supernatant interleukin (IL)-13 and interferon (IFN)-γ from whole blood cultured with SEA or SWAP……………………………….……73

LIST OF TABLES

Table 4.1: Characteristics of study participants at baseline 42

Table 5:1 General characteristics of the study participants at baseline and 1 year post-treatment...63

ACRONYMS AND ABBREVIATIONS

ANOVA	Analysis of ovariance
APC	Allophycocyanin
CAA	Circulating anodic antigen
CCA	Circulating cathodic antigen
CD	Cluster of differentiation
CDC	Centres for disease control and prevention
CGHR	Centre for global health research
CLTS	Community led total sanitation
DALYs	Disability adjusted life years
DLC	Dynein light chain
ELISA	Enzyme-linked Immunosorbent Assay
EPG	Eggs per gram
FACS	Fluorescence activated cell sorter
FBS	Foetal bovine serum
FcεR	Fragment crystallisable epsilon receptor
FITC	Fluorescein isothiocyanate
FMO	Fluorescence minus one
FOXP3	Forkhead helix transcription factor 3
FSC	Forward scatter
GCP	Good clinical practice

HIV	Human immunodeficiency virus
HRP	Horse radish peroxidase
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IQR	Inter-quartile range
KEMRI	Kenya medical research institute
MDA	Mass Drug Administration
MoPHS	Ministry of Public health and sanitation
MPDP	Merck praziquantel donation program
NTD	Neglected tropical diseases
ODF	Open defaecation free (area)
PBMC	Peripheral blood mononuclear cell
PZQ	Praziquantel
RPMI	Roswell Park Memorial Institute Media
SBD	School based treatment
SCI	Schistosomiasis Control Initiative
SEA	Soluble egg antigens
SWAP	Soluble worm antigenic preparation
SSA	Sub-Saharan Africa
SSC	Side scatter
TAL	Tegumental Allergen-Like protein
TGF	Transforming growth factor
T_H	Helper T lymphocyte
TMB	Trimethyl benzydine
TNF	Tumour necrosis factor
Treg	Regulatory T lymphocyte
WHA	World Health Assembly

WHO World Health Organization

ABSTRACT

The areas around Lake Victoria in Western Kenya are endemic for schistosomiasis mansoni,a disease that infects more than 200 million people worldwide, majority of whom are children. Kenya and other countries where the disease is endemic are currently carrying out mass drug administration using praziquantel targeting school children for morbidity control and reduction in prevalence. Prevalence and intensity of infection of this disease in endemic areas tends to increase with age, plateauing and eventually falling from early adulthood. It is hypothesised that this age associated prevalence and intensity of infection is a reflection of a similar age-dependent development of protective anti-schistosome immune responses. The immunological age profile of children in western Kenya infected with *Schistosoma mansoni* has never been determined. Mass drug administration (MDA) with praziquantel was hypothesised to alter this profile. This study therefore sought to profile immune responses associated with protection against re-infection of schistosomiasis in 6-17 year old school children, and the effect of one round of mass drug administration (MDA) with praziquantel on these responses in pre- and early teenage years. It was a repeated cross-sectional study in which a total of 387 participants were recruited, 288 at baseline and 99 1 year later from all classes of primary and secondary schools in Asembo area of Rarieda Sub-county of Siaya County in Western Kenya. Each consenting participant provided 3 consecutive stool samples for diagnosis of

schistosomiasis by Kato-Katz and about 10 ml of blood for immunological assays. Anti-schistosome crude soluble worm antigen preparation (SWAP) and soluble egg antigen (SEA) and recombinant tegument allergen-like (TAL) antibodies were assayed by ELISA. Parasite antigen-specific cytokines were also assayed from whole blood culture supernatants by ELISA, while CD19+CD23+ B lymphocytes were determined by flow cytometry.Data was analysed using GraphPad Prism software, version 5. Parametric data was analysed by ANOVA and Tukey's multiple comparison post-test. Kruskal Wallis test and Mann Whitney U tests were used for non-parametric data. While most of the anti-SWAP and anti-SEA antibodies did not differ significantly across the different age-groups, anti-SEA IgE and total IgG were significantly different across the age-groups ($P<0.0001$). Subsequent post-hoc analysis showed that the 9-11 and 12-14 year old age groups had higher anti-SEA IgE (median, 433.8 arbitrary units (AU); Interquartile range, IQR, 692.0 AU and median, 268.1; IQR 497.9 AU respectively) compared to the 15-17 year olds (median, 90.3; IQR 160.0 AU; $P<0.0001$. The pattern for anti-SEA IgG was quite the opposite, with 9-11 year olds producing less IgG (median, 20.6; IQR, 120.2 AU) than 15-17 year olds (median, 201.2; IQR, 488.6 AU; $P<0.0001$). Moreover, 12-14 year olds had less IgG antibody (median, 54.9; IQR, 177.1 AU) than 15-17 year olds (median, 201.2; IQR 488.6 AU; $P<0.0001$). The rest of the antibodies assayed, CD23+ B cell levels did not differ significantly with age ($P>0.05$). Anti-*Schistosoma mansoni*-Tegument Allergen-like (SmTAL) antibodies similarly did not differ significantly across the age groups ($P>0.05$). In post-treatment comparisons, anti-SEA IgE levels were higher in the combined post-treatment group in contrast to anti-SEA IgG. In addition, the post-treatment schistosomiasis egg positive group had higher anti-SEA IgG4 levels (median, 117.4; IQR, 553.2 AU; $P<0.0001$) compared to the schistosomiasis negative

group (median, 3.7; IQR, 37.2 AU). The same IgG4 levels against SmTAL-2 antigen was higher in the pre-treatment group (median, 16.0; IQR, 183.6 AU) relative to schistosomiasis egg negative post-treatment group (median 0.0; IQR, 1.6 AU; *P*<0.05). Anti-SmTAL-5 IgE was similarly reduced post-treatment. A decline-post-treatment was also observed with the proportion of CD23+ B cell levels post-treatment (mean, 61.7; SEM, 1.2) relative to pre-treatment proportions (mean, 67.4; SEM, 0.8 %; *P*<0.05) . For cytokines, post-hoc analysis for IL-5 showed that the schistosomiasis negative post-treatment group had higher mean levels of this cytokine (mean, 84.9; SEM, 32.1 pg) compared to mean baseline levels (mean, 6.4; SEM, 2.1 pg; P<0.0001) and schistosomiasis positive post-treatment group (mean, 21.7; SEM, 7.3 pg; P<0.001). Soluble egg antigen stimulated mean IL-13 levels were higher in the combined post-treatment group (mean, 137.8; SEM 41.7 pg) compared to mean baseline levels (mean, 19.9; SEM, 4.0 pg; P<0.05). Similarly, mean IL-13 levels produced in response to SWAP were higher in the combined follow-up group (mean, 127.0; SEM, 28.3 pg; P<0.001) and in the schistosomiasis positive post-treatment group (mean, 134.6; SEM, 42.3 pg; P< 0.05) relative to mean baseline levels (mean, 29.3; SEM, 6.2 pg) Taken together, these results are indicative of the complex nature of immune responses in individuals of comparable demographic and epidemiological conditions. It is clear from these results that a single treatment of schistosomiasis with praziquantel may not result in augmented anti-schistosome immune responses that correlate to resistance to reinfection. It remains possible, however, that multiple rounds of annual mass drug administration may have beneficial effects leading to the development of resistance in children, necessitating further longitudinal research in this area.

CHAPTER ONE: INTRODUCTION

1.1 Background

Schistosomiasis, commonly known as bilharzia is a disease of the tropics caused by blood dwelling parasites of the genus *Schistosoma*. Five species of *Schistosoma* are known to infect human beings: *Schistosoma mansoni*, *Schistosoma japonicum*, *Schistosoma mekongi*, *Schistosoma haematobium*, and *Schistosoma intercalatum*(Colley *et al*. 2014).Three of the five species are more widespread. These are *S. mansoni* which is transmitted by *Biomphalaria* snails and causes intestinal and hepatic schistosomiasis; *S. haematobium*, transmitted by *Bulinus* snails and causing urinary schistosomiasis and *S. japonicum*, transmitted by the amphibian snail *Oncomelania* and causing intestinal and hepatosplenic schistosomiasis. *Schistosoma japonicum* is a zoonotic parasite that infects multiple other mammals such as cattle, dogs, pigs and rodents. *Schistosoma mansoni* has also been reported to infect rodents and primates(Gryseels *et al*. 2006)

Adult schistosome worms are between 7-20 mm in length. They have a cylindrical body with two terminal suckers, a complex protective tegument, a blind digestive tract and reproductive organs. The worms have separate sexes with the male forming a groove or gynaecophoric channel in which the long slender female dwells. The worms live aspairs within the perivesical for *S. haematobium* or mesenteric venous plexus for the other species. They feed on blood and globulins by anaerobic respiration and since they lack an anus, regurgitate debris into the host's bloodstream. Some components of the wastes regurgitated are targets for urine and blood-based rapid diagnostic tests(Colley *et al*. 2014).

1.1.1 Life cycle of schistosomes

Human infection by schistosomes occurs when an individual comes in direct contact with fresh water that harbours the free swimming larval form of the parasite called cercariae. Cercariae are shed by infected fresh water snails. The larvae possess a forked tail and are non-feeding, utilizing their endogenous nutrients for energy production. The implication is that they must come in contact with the skin of their primary host within 12-24 hours after emerging from the snail(Gryseels *et al*. 2006). On contact with the skin, percutaneous penetration occurs by both mechanical activity and by the production of proteolytic enzymes. As they penetrate, the cercariae shed their tails, becoming schistosomulae and enter the lymphatic system from where they move to the blood circulatory system which transports them to the lungs. After a short stay in the lungs, the developing schistosomulae then re-enter blood circulation for transport to the portal or vestibule circulation to complete the life cycle. It is from here that the schistosomulae mature, couple up for reproduction and migrate to their perivescular or mesenteric destination where egg production occurs(Colley *et al*. 2014).

It takes about 4-6 weeks from infection for the larvae to mature into adult male and female worms and before egg production commences. Egg production continues for the rest of their life, which averages 3-10 years but may also be as long as 40 years(Warren *et al*. 1974; Chabasse *et al*. 1985). The females produce hundreds to thousands of eggs per day depending on the species. The tough-shelled egg contain a ciliated miracidium larvae that secrete proteolytic enzymes. These enzymes help them to cross the endothelium and basement membrane of the vein and epithelium of the intestine or bladder to be shed with faeces or urine and complete the life cycle. The

mechanism by which eggs migrate across the venous and intestinal walls is not clearly understood although there seems to be an immunological component to it. This is based on the observation that egg excretion in immunocompromised mice is minimal(Doenhoff 1997).A correlation between diminished egg excretion and decreased $CD4^+$ T cell count in *S. mansoni*-infected HIV positive patients has also been reported*(Karanja et al*. 1997).

The miracidia lavae hatch from eggs once they come in contact with fresh water. Before this, eggs may remain viable for up to 7 days.The larvae then, using light and chemical stimuli, search for their respective fresh water snail which serves as the intermediate host. Inside the snails, the miracidia larvae multiply asexually into multicellular mother and then daughter sporocysts and later into cercarial larvae to continue the life cycle (Fig 1.1)

1.2 Schistosomiasis distribution

1.2.1 Global schistosomiasis distribution

Schistosomiasis is the second most prevalent severe parasitic disease after malaria, with active transmission reported in 67 countries where its presence has been reported(Engels *et al*. 2002). The three major schistosome parasites of humans are *S. haematobium*, *S. mansoni* and *S. japonicum* and these three are prevalent in parts ofAfrica, the Middle East, South America, China, south-east Asia and the Philippines. Specifically, *Schistosoma mansoni* is found in 54 countries, including part of the Arabian Peninsula, northern Africa, sub-Saharan Africa, Brazil and some Caribbean islands. *Schistosoma haematobium* is endemic in 53 countries in the Middle East and most of the African continent, while *S. japonicum* is endemic in China, Indonesia and the Philippines (Fig 1.2). It has been estimated that globally 779 million people are at risk of infection and those infected are about 207 million(Steinmann *et al*. 2006).

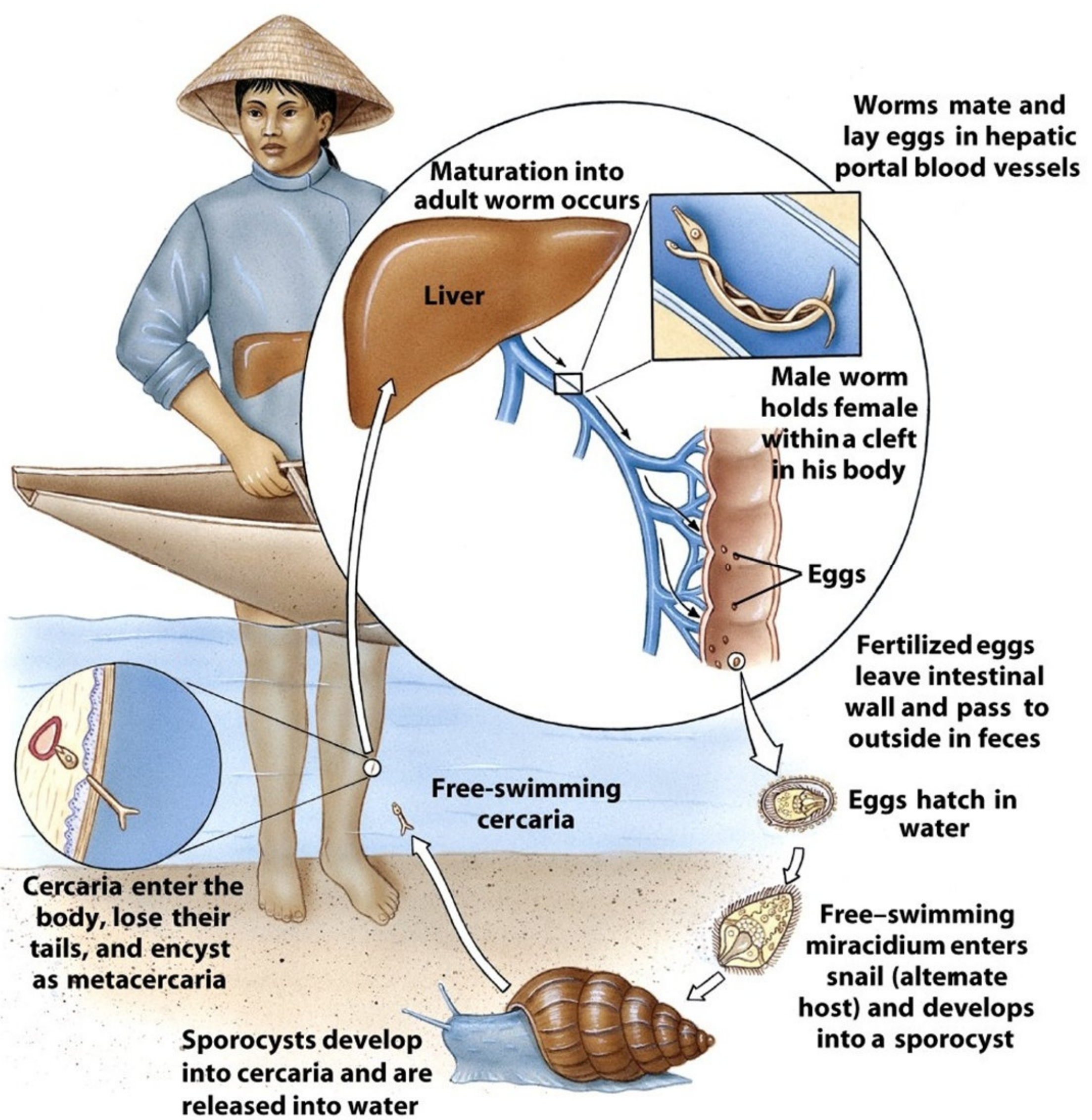

Figure1.1:Life cycle of *S. mansoni,* (Adopted from http://www.ppdictionary.com/parasites/schistosomes_main.jpg)

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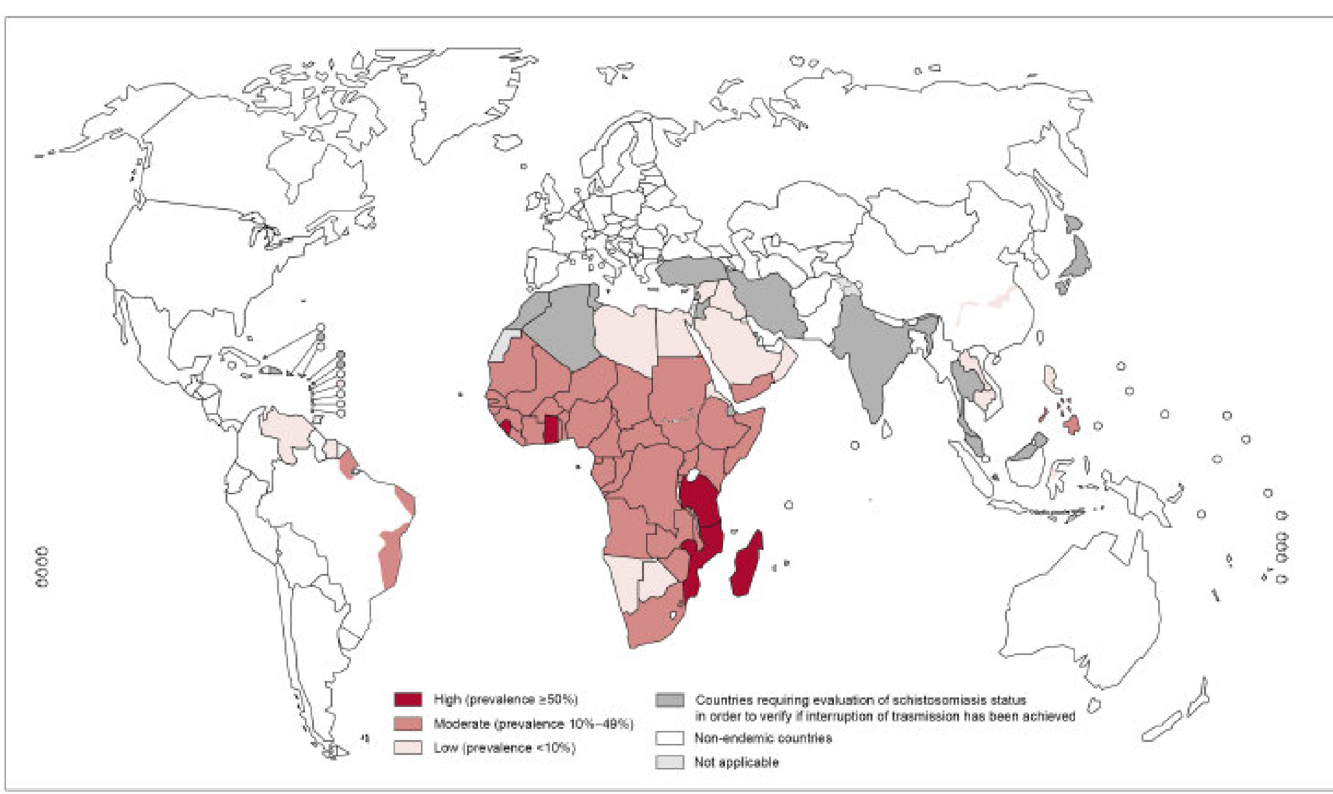


Figure 1.2: Global distribution of schistosomiasis (courtesy of WHO).

1.2.2 Schistosomiasis in Kenya

Schsistosomiasis is endemic in many parts of Kenya, particularly in the Coastal and Lake Victoria region. Other areas of endemicity are Mwea and Machakos in Eastern Kenya and Nyando basin in Western Kenya, places that undertake irrigation farming (Fig 1.3). *Schistosoma haematobium* and *S. mansoni* are the two common species in the East African region. *Schistosoma haematobium* is more prevalent at the coast while prevalence of *S. mansoni* in the Lake Victoria region is found to increase with proximity to the lake. There are also cases of*S. haematobium,* withprevalence increasing with distance from the lake in contrast to *S. mansoni*(Handzel *et al*. 2003). Around Lake Victoria in Kenya,men and school age children are most impacted by schistosomiasis due to the high frequency of water contact as a result of activities such as swimming, bathing, washing and fishing (Handzel *et al*. 2003). Kenya's

Ministry of Public Health and Sanitation (MoPHS) reportsindicate that 6 millionKenyans are at risk of infection, and has proposed interventions, especially preventive chemotherapy, targeting school children in 39 districts where the disease has been confirmed to occur (Ministry of Public Health and Sanitation Kenya 2011).

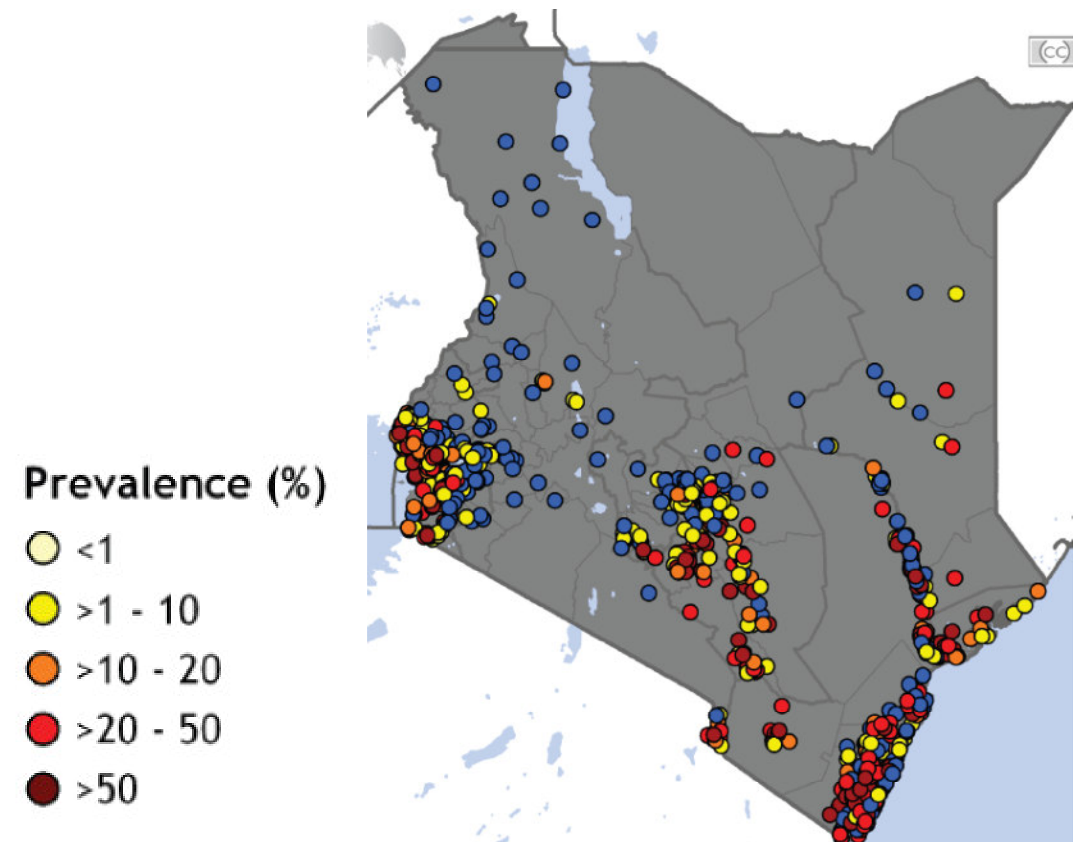


Figure1.3: Prevalence of schistosomiasis in Kenya(Courtesy of the Wormy World Project, www.thiswormyworld.org)

1.3 Economic importance of schistosomiasis

Schistosomiasis is second to malaria as a leading parasitic disease in of terms severity and number of people infected and those at risk of infection. It was initially estimated that the disease causes an annual loss of between 1.7 and 4.5 million disability adjusted life years (DALYs)(Steinmann *et al*. 2006). This is a time-based health-outcome measure that includes weights for time spent in less-than-perfect health, it is the sum of years of life lost and years of life lived with disability developed under the

World Health Organisation's(WHO's) Global Burden of Disease program. More recent analyses have challenged these estimates, claiming serious underestimation. Newer estimates give figures of 3.31 million DALYs (Hotez *et al*. 2014). While these estimates still rank below other more lethal diseases such as malaria, HIV and tuberculosis, schistosomiasis-related disability is borne daily by over 200 million people worldwide, the majority of whom live in poverty prone areas of sub-Saharan Africa(Steinmann *et al*. 2006).

There are several clear morbidities associated with *S. mansoni* infection including hepatosplenomegally, periportal fibrosis, portal hypertension, oesophageal varices and haematemesis(Vennervald and Dunne 2004). The majority of individuals infected with this disease do not experience these morbidities. They are however likely to suffer one or more of theless obvious but significant disabilities such as growth stunting, anaemia, abdominal pain, exercise intolerance, cognitive impairment, poor school performance and lowered work capacity (King *et al*. 2006). With the highest prevalence and infection intensities of schistosomiasis being in school-age children, adolescents and young adults, negative effects on school performance is a serious problem (Steinmann *et al*. 2006). The debilitation caused by untreated infections also impede social and economic development in areas of high prevalence undermining efforts put in place to alleviate poverty, one of the key targets of the millennium development goals that were set by the United Nations (UN). Many lives are also lost directly as a result of schistosomiasis, with estimations giving a figure of 130,000 per year for *S. mansoni* alone in sub-Saharan Africa (van der Werf *et al*. 2003). However,it is encouraging to note the increasing attention that schistosomiasis and other neglected tropical diseases (NTDs) are now getting from WHO andgovernments of countries where these diseases are endemic. The approach being

taken is morbidity reduction and possibly transmission control through mass chemotherapy.

1.4 Schistosomiasis diseasepathogenesis

1.4.1 Acute disease

In newly infected patients, especially those travelling to endemic areas for the first time, the acute phase of schistosomiasis is manifested as Katayama syndrome, a systemic hypersensitivity reaction against the migrating schistosomulae and eggs, and manifests within two to twelve weeks after a primary infection(Ross *et al*. 2007). The percutaneous penetration of cercariae in newly infected persons can also trigger a rash on the skin called cercarial dermatitis. Symptoms for the systemic reaction include fever, fatigue, myalgia, malaise, non-productive cough and eosinophilia(Ross *et al*. 2007; Gryseels *et al*. 2006). In some patients, abdominal symptoms may develop later, caused by the migration and positioning of the mature worms. In most patients these symptoms ameliorate after 2-10 weeks of infection. Other individuals develop persistent and more serious disease(Ross *et al*. 2007; Gryseels *et al*. 2006).

1.4.2 Chronic disease

In the chronic phase, schistosomiasis mansoni may manifest in two different clinical forms depending on an individual. It may cause a variety of subtle morbidities under the intestinal form of the disease, which affects most individuals, or it may develop into the severe hepatosplenic form in some few cases due to suboptimal immunoregulation of their immune responses to antigens from parasite ova (Colley *et al*. 2014). In intestinal schistosomiasis, migration of eggs through the intestinal wall may provoke mucosal granulomatous inflammation, microulceration and superficial bleeding. The most common symptoms of this form of disease are chronic or

intermittent abdominal pain and discomfort, loss of appetite and diarrhoea with or without blood. The frequency and severity of the symptoms in infected people is related to intensity of infection (Gryseels *et al*. 2006). The hepatosplenic form of the disease is characterised by hepatosplenomegaly, periportal fibrosis, portal hypertension, ascites, oesophageal varices, collateral circulation, haematesis, and death if not treated(van der Werf *et al*. 2003; King, Dickman, and Tisch 2005). In thechronic phase of the disease, there is hepatic inflammation in response tothe trapped eggs in the periportal spaces of the liver leading to hepatosplenomegaly in up to 80% of infected children and adolescents(King, Dickman, and Tisch 2005). Hepatic inflammation is less common and intense in adults, butmassive deposition of collagen in the periportal spaces is more likely to occur leading to hepatic fibrosis. The fibrosis process takes 5 to 15 years and may eventually lead to portal hypertension and bleeding from gastro-oesophageal varices which is the most common cause of mortality in schistosomiasis(Richter *et al*. 1998).

1.5 Diagnosis of schistosomiasis

The standard and preferred method for diagnosis of schistosomiasis mansoni is microscopy using Kato Katz technique. It involves detection of parasite eggs in stool samples. The eggs are fairly easily detected and identified in faeces due to their size and lateral spine (Fig 1.4).For detection of mild and light infections, concentration methods such as sedimentation in a glycerine solution or centrifugation in formalised ether are needed. The Kato-Katz method(Katz *et al*. 1972) is the most commonly used method of diagnosis since it allows quantification of the infection by egg counts, usually reported as eggs per gram of faeces (EPG). It is a rapid and simple technique that requires 40 to 50 mg of faeces. It is widely used in field studies and national control programmes(Feldmeier *et al*.1993).The major shortcoming for this technique

is that it is not able to detect infection in early stages, that is, before egg production commences. It may also fail to detect eggs in light infections.

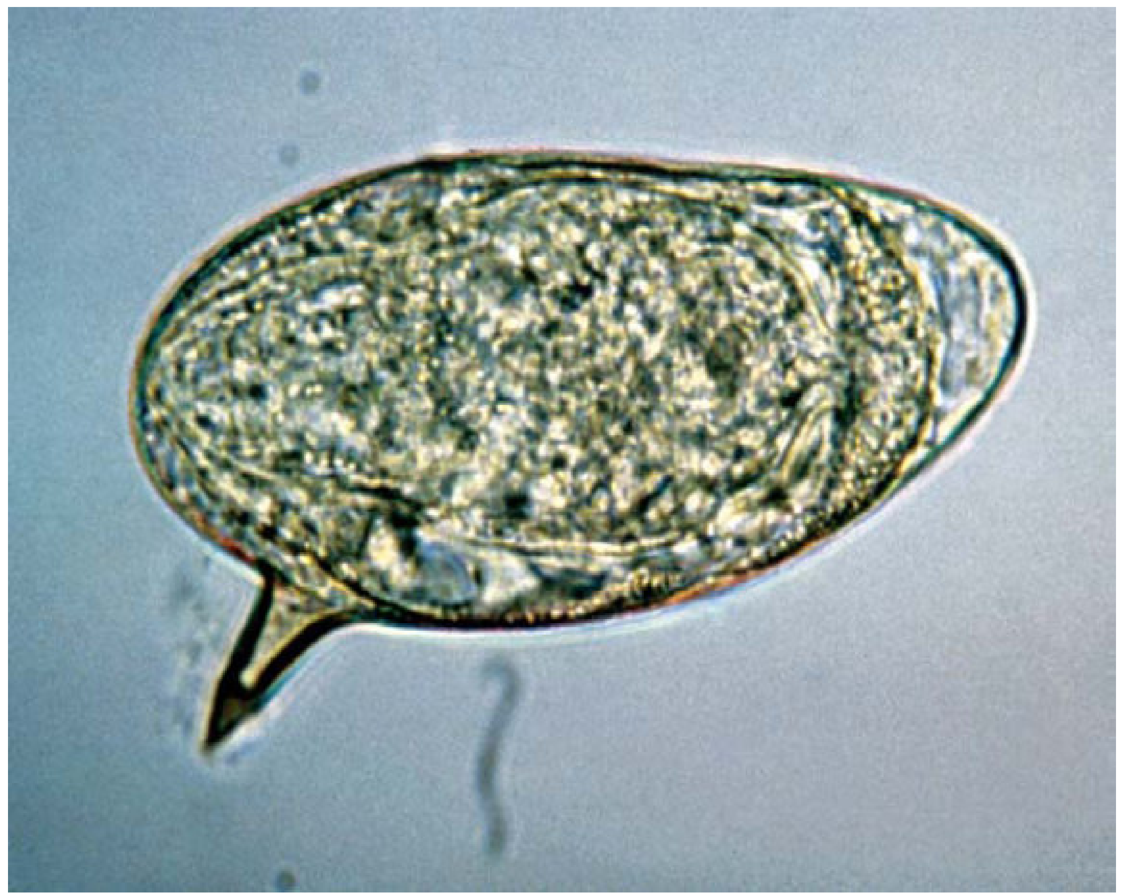


Figure1.4: ***Schistosoma mansoni*** **egg(Adapted from(Ross** ***et al.*** **2007)**

Diagnosis can also be done using antibody detection techniques that are preferable in a few specific circumstances such as diagnosis in patients not excreting eggs and in field studies for defining regions of low-level endemicity where individual patients have low egg burdens. They may also be useful in determining re-emergence of infection in a region following an apparently successful control program and for diagnosis in asymptomatic travellers (Ross *et al.* 2002). While antibody based assays are quite sensitive, they cannot distinguish past infection from active infection(Colley *et al.* 2014). They can also cross-react with other helminthes and are not easily applicable under field conditions(Gryseels *et al.* 2006). There are also tests to detect

schistosome antigens, such as the point-of-contact (POC) circulating cathodic antigen (CCA) and circulating anodic antigen (CAA) tests. The POC lateral flow cassette assay is used for testing parasite antigens for *S. mansoni* in urine samples and is now commercially available. Molecular techniques can also be used to for the detection of schistosome DNA in stool specimens(Colley *et al*. 2014).

1.6 Control of schistosomiasis

1.6.1 Chemotherapy using Praziquantel

Praziquantel (PZQ) is the single most efficacious drug for the treatment of all schistosome species. It was first synthesised by scientists at the German chemical company, E Merck in 1977 (Dömling and Khoury 2010). It is a synthetic tetracyclic tetrahydroisoquinoline derivative and is part of WHO's 'Essential Medicines' list. It is commonly available in the market as 600 mg tablets. It is available to Ministries of Health through a donation by Merck-Serono through the WHO. The recommended standard regimen is 40 mg/kg body weight in a single dose. The actual mechanism of action of the drug on adult worms is unknown. It has however been reported to be associated with calcium influx and muscular contractions (Dömling and Khoury 2010). It has mild side-effects which include nausea, vomiting, malaise and abdominal pain and is safe for the treatment of children and pregnant women. The drug is ineffective on eggs and immature worms(Botros *et al*. 2005),so follow-up treatment may be required 4-6 weeks later. A single dose of the drug is nonetheless still quite effective since 70-100% of patients cease to excrete eggs after treatment (Renganathan 1998), although this level of efficacy may be related to the insensitivity of the Kato-Katz assay to detect low levels of eggs in the stool and when the more sensitive POC-CCA assay is used the level maybe approximately 50% (Mwinzi *et al*. 2015).

1.6.2 Other drugs for treatment of schistosomiasis

Oxamiquine is another drug that is effective in treatment of *S. mansoni* infection. Unlike PZQ, this drug is not effective against all species of *Schistosoma*. It also has more pronounced side-effects such as drowsiness, sleep induction and epileptic seizures. This drug hasvery limited availability in the market(Dömling and Khoury 2010). Artemether, which is a well known anti-malarial drug, has been shown to be effective against immature stages of schistosomes (Utzinger et al. 2000). A combination of praziquantel and artemisinin derivatives could be more effective in enhancing rates of cureand in the control of infection (Colley *et al*. 2014). More research is however needed to determine dosage, formulation and drug interactions before combination of these drugs can be adopted as a standard treatment for schistosomiasis. The potential risk of inducing resistance to artemisinin derivatives in malarial parasites should be of concern when considering its use for schistosomiasis control in areas where malaria is endemic (Utzinger *et al*. 2000).

1.6.3 Other control strategies

China and Philippineshave successfully combined the elimination of the amphibian snails with treatment of infected humans and selected animal hosts to successfully control schistosomiasis(Fenwick and Webster 2006). Egypt has also been very successful by initially focusing on transmission reduction through snail control supplemented by chemotherapybetween 1953 and 1985; and later focused on morbidity control through MDA using PZQ (Barakat 2013). Current control strategies are focused on evidence-based chemotherapyimplemented depending on disease prevalence. In areas of low endemicity, the strategy recommended is treatment of only positive diagnosed cases, while in areas of moderate to high prevalence, mass treatment of all school-aged children is advised(Fenwick and Webster 2006).Control

of the vector snails which is undoubtedly a daunting task would also be an important component of schistosomiasis control.However, the eradication of this disease will require an integrated, sustained and inclusive approach involving public education and community sanitation improvement measures. Specific measures to halt reinfection should include provision of safe clean water in communities where the disease is endemic, construction of toilets and latrines, and public education on the importance of maintaining good sanitation and high standards of hygiene. One of the programs that is being implemented in different parts of the developing world that encompasses some of these measures is Community-Led Total Sanitation (CLTS) program. This is an integrated approach to achieving and sustainingopen defecation free (ODF) statusin a given community or village as a way of interrupting the life-cycle of the parasite in areas where the disease is endemic (Kar and Chambers 2008). The main advantage of such program is assured sustainability due to community participation and ownership of these disease control measures.

CHAPTER TWO: LITERATURE REVIEW

2.1.1Schistosomiasis infection in children

Prevalence and intensityof infection of schistosomiasis in endemic areas increases with age, peaking between the ages of 10 to 15 years. Standard age prevalence curves which are based on egg excretion show a gradual decline of prevalence over years with infection intensity sharply declining following a peak in the mid teen years(Kabatereine *et al*. 1999). This decline in prevalence and infection intensity has been attributed to development of acquired protective immunity to new infection. Immunity development coincides with the time when worms from early infections begin to die, given their lifespan of about 5 to 10 years(Woolhouse and Hagan 1999). It is theorised that critical schistosome antigens previously un-encountered by host immune cells are released upon death of worms eliciting protective immune responses that result in increased resistance to new infections(Fulford *et al*. 1995; Woolhouse and Hagan 1999).

2.2 Immune responses during acute and chronic stages

Research in animal models have shown a predominantly T_H1-type immune response with elevated production of IFN-γduring the first three to five weeks of infection. As egg production begins, T_H1 components decline with an associated emergence of a strong T_H2-type response(Pearce and MacDonald 2002).Many murine studies report a mixed cytokine profile during acute phase of the disease with the cytokines mostly reported being IFN-γ, IL-10 and IL-5(Montenegro *et al*. 1999).Peripheral blood mononuclear cells (PBMCs) from patients with acute disease have higher proliferative responses to parasite soluble egg (SEA) antigen and soluble worm antigen preparation (SWAP) than those from patients with chronic disease(Malaquias *et al*. 1997).

The immune response in the chronic phase of the disease is marked by a predominantly T_H2 cytokine profile although a few studies have reported a T_H1 profile (Mwatha *et al*. 1998). This underscores the variable nature of the immune response in chronic patients, which is a mix of both T_H1 and T_H2 cytokines(Jesus et al. 2004).The T_H2 response against egg antigens has dual effects on the host. While critical for protection of the host they are also associated with host morbidity.Polarization toward T_H2 requires the presence of IL-4. In schistosomiasis infected mice, there is no strong polarization of CD4 T cell responses toward T_H2 in the absence of IL-4. Additionally, these mice are not able to survive for long after commencement of egg production (Brunet *et al*. 1997; Fallon 2000). While T_H2 response serves the beneficial role of host protection against severe morbidity, IL-13, another cytokine made by T_H2 cells, is implicated in the severe fibrosis that followsgranuloma formation around schistosome eggs. This cytokine is associated with the production of the enzyme arginase in myofibroblasts leading to increased collagen production and fibrosis (Oliveira*et al*. 2006).

Development of T_H2 responses during schistosomiasis infection from naive $CD4^+$cells is brought about by several cells of the innate immune system.Basophils, eosinophils and mast cells are potential innate sources of cytokines such as IL-4 which are associated with polarization of T_H2 responses.Dendritic cells (DC) play a major role in the polarization of T_H2 responses during helminth infections.DCs activated by eggs or soluble antigens secreted by eggs prime T_H2 polarization(Everts *et al*. 2009). One of the soluble egg secretory products shown to be involved in T_H2 induction is omega-1/T2 ribonuclease(Everts *et al*. 2009; Steinfelder *et al*. 2009) demonstrated that Omega-1 was able to instruct human DCs to potently prime T_H2 polarised

responses from naive human $CD4^+$ cells in vitro.Control of pathology during the course of murine schistosomiasis infection is also linked to the activity of alternatively activated macrophages which are important immune regulatory cells of the innate arm of the immune system(Herbert *et al*. 2004). Alternative activation of macrophages is linked to T_H2 responses and is also associated with increase in collagen synthesis and fibrogenesis(Burke *et al*. 2009; Barron and Wynn 2012).

Alongside these T_H2 immune responses during chronic schistosomiasis are other phenotypes of helper T cells. Regulatory T cells (Treg) play a very significant role in modulation of both T_H1 and T_H2 responses, thus helping to prevent severe pathology. These cells are characterized by production of IL-10 and can either be naturally occurring $CD4^+$ $CD25^+$ T cells or $CD4^+$ T cells induced in response to parasite antigens(Hesse et al. 2004). A marker used to identify these cells is forkhead helix transcription factor 3 (FOXP3), a key transcription factor inthe development of Treg cells (Vignali *et al*. 2008; Burke *et al*. 2009). The regulatory role of these cells has mainly been attributed to production of inhibitory cytokines such as IL-10 and transforming growth factor-beta (TGF-β) (Hesse *et al*. 2004; Vignali *et al*. 2008). Besides Treg, other immune cells have been demonstrated to produce IL-10 with inhibitory activity. They include $CD8^+$ T cells, monocytes and mast cells. While this cytokine is critical in controlling acute granulomatous inflammation in mice, it has also been linked with blockage ofdevelopment ofresistance against reinfection after treatment(Wynn *et al*. 1998; Wilson *et al*. 2011). The cytokine is also key to the switch from T_H1 to T_H2 during early stages of infection with the onset of egg laying.

The $T_H17$lineage is also involved in schistosomiasis immune response. This subset of cells produces IL-17 family of cytokines which are IL-17A, B, C, D, E and F, some of which are associated with pathology and susceptibility to schistosomiasis reinfection

in mice. Interleukin-17 production in mice is associated with increased hepatic pathology and susceptibility to reinfection while IL-17E (IL-25) amplifies T_H2 responses (Zaccone *et al*. 2008).

Studies involving animal models have contributed quite substantially to what is known about schistosomiasis disease.The disease takes two divergent courses of progression in the rat and in the mice. Schistosomiasis infection in the rat is halted before the parasite reaches sexual maturity and therefore before egg production. There is a strong immunity formed against reinfection(Capron and Capron 1986).The protective response has been demonstrated to be mainly humoral, involving antibody-dependent cell-mediated cytotoxicity. In vitro studies showed the participation of IgG2a and IgE in the killing of schistosomula, in the presence of eosinophils, and enhanced by mast cells (Grezel *et al*. 1993). Mice on the other hand are permissive hosts in which the infection proceeds to full pathology unchecked. Mice do however develop resistance to reinfection when vaccinated using irradiated cercariae. The mechanism of protection in mice is quite divergent to what is observed in the rat model, involving a T_H1 inflammatory response that stops migration of schistosomulaeand kills larvae (Mountford *et al*. 1996; Capron and Dessaint 1985).

2.3 Morbidity in schistosomiasis mansoni

Morbidity in schistosomiasis mansoni infection isfirst manifested as an itch or 'swimmers rash' at the point of cercarial penetration into the skin and accompanying immune response. This is however occurs mostly in newly infected persons.The main cause of morbidity in infected persons during acute and chronic phases of the disease is as a result of host inflammatory immune response to parasite eggs that become trapped in hepatic sinusoids. The development and level of morbidity in an individual depends on several factors. These include: degree and length of exposure, intensity of

infection, co-infections, parasite and host genetics,the host immune response to the parasite and the interplay between regulatory and inflammatory responses (Vennervald and Dunne 2004; Colley and Secor 2004). In intestinal schistosomiasis, morbidity involves the intestinal tract, manifesting as diarrhoea, abdominal pain and intermittent blood in stool. Some patients develop hepatosplenic disease characterised by liver and spleen enlargement. This results from vascular and granulomatous changes associated with trapped eggs which occurs in some individuals who poorly immunoregulate responses to the trapped eggs (Cheever 1968).

Formation of granulomas is mediated by $CD4^{+}$ T cells and involves cellular infiltration, mainly of eosinophils, macrophages, fibroblasts and lymphocytes(Pearce and MacDonald 2002). The granulomas resolve with death of eggs, leaving fibrotic plaques in the liver. It is the resulting fibrosis that often leads to portal hypertension. While granulomas themselves are pathogenic, they also serve a host-protective role. Mice tolerized against *S. mansoni* egg antigens do not form granulomas after infection but suffer severe liver damage(Fallon and Dunne 1999). The granulomas sequester egg-secreted toxins from hepatocytes. In addition, egg-antigen-specific antibodies may have a neutralising effect on the toxins(Fallon and Dunne 1999).

Several cytokines are associated with egg granuloma formation. In a study that evaluated the cytokine profile in schistosomiasis patients developing hepatic fibrosis in prehepatosplenic and early hepatosplenic stages, cytokines IL-5, IL-10 and IL-13 were associated with hepatic fibrosis. IL-5 and IL-13 showed the strongest association with severe hepatic fibrosis(Jesus *et al.* 2004). Other studies have reported an association between low IFN-γ and TNF-α with severe hepatic fibrosis(Booth *et al. 2004;* Henri *et al.* 2002). In another study involving severe combined

immunodeficient mice, their injection with TNF-α allowed the development of granulomatous lesions around eggs (Amiri *et al*. 1992). There is agreement from most studies including those involving mice about the protective role of IFN-γ in severe fibrosis of the liver(Hoffmann *et al*. 2000; Chiaramonte *et al*. 2001).

There are many other complications that may arise from granuloma formation subsequent to fibrosis and portal hypertension. These include splenomegaly, portacaval shunting, ascites, gastrointestinal bleeding and anaemia(Gryseels *et al*. 2006). Further complications which have been reported as resulting from anaemia include growth retardation, wasting in adults and cognitive impairment in children (Ross *et al*. 2002).

2.4Schistosomiasis control using mass drug administration

Praziquantel is the most widely used and effective drug for the treatment of schistosomiasis. It is also the drug of choice in the implementation of the World Health Assembly resolution 54.19 that calls for the regular treatment of all school-age children at risk of contracting schistosomiasis (WHO 2001).In 2006, the target population was expanded further to include other high risk groups in endemic areas such as older preschool children, adults considered to be at great risk such as pregnant and lactating mothers, those engaged in occupations that involve exposure to cercariae infested water and whole communities living in areas of high endemicity (WHO 2006). This expansion was necessitated by the realisation that besides school children, there are other population groups that are at risk of developing morbidity, especially older pre-school age children in areas of high transmission. Additionally, there has been an increased affordability and availability of praziquantel in the market as well as donation of PZQ for mass drug administration (MDA) programs (Savioli *et al*. 2009).

There has been steady increase in the number of people treated for schistosomiasis worldwide since 2006 under various programmes involving partnerships between governments and other partner organisations. One of the ongoing programs is the Schistosomiasis Control Initiative (SCI) that is operating in 7 African countries. It is acollaboration between government ministries and the Bill and Melinda Gates Foundation through the Imperial College London. Together with other initiatives and support from WHO, the number of people treated worldwide rose from 12.4 million in 2006 to 33.6 million in 2010 (271% increase), according to figures released by WHO (WHO 2012). The report further states that the total number of people needing preventive chemotherapy globally for 2010 was 237 216 451, of which 108 950 695 were school-age children in 51 out of 71 countries where the disease is endemic.Forty of these countries are in Africa with 83.4% of those treated in 2010 also being on the continent.The majority (73.8%) of individuals treated were school-age children. Overall, only 31% of people needing treatment were actually treated(WHO 2012).

Kenya had for a long time not implemented a schistosomiasis preventive chemotherapy program at national level despite being one of the countries with high prevalence levels.The disease is endemic in 36 districts mostly found in three major regions: Coast, Lower eastern and Lake Victoria Basin. *Schistosoma mansoni* is present in most parts of Lower Eastern and Lake Victoria regions(Ministry of Public Health and Sanitation Kenya 2011). The Kenyan government through the then MoPHS launched a nationwide campaign to control Neglected Tropical diseases (NTD's) through MDA targeting school age children in November 2011(WHO Kenya 2011). Schistosomiasis was for the first time be included in this deworming campaign. The launch was marked with the release of the 'National Multi-year strategic Plan for

control of Neglected Tropical Diseaeses 2011-2015', a five year integrated program to co-ordinate efforts to control NTDs. The plan has a goal of 100% MDA in all communities where schistosomiasis is endemic. These communities are spread across 36 districts. The program aims to reduce morbidity of schistosomiasis to levels where it is no longer a public health problem through MDA using PZQ(Ministry of Public Health and Sanitation Kenya 2011).

Besides controlling morbidity, treatment also seems to increase resistance to reinfection. Several previous studies done in our laboratory have reported increased immune responses that correlate with resistance to reinfection after repeated treatments. In an earlier study involving adult car washers occupationally exposed to schistosomiasis infection, some individuals in the cohort were highly susceptible to reinfection despite equal exposure to those at a lower susceptibility at the beginning of the study. Some of these individuals became increasingly resistant to reinfection after multiple subsequent retreatments after reinfections(Karanja *et al*. 2002). Similar findings were made in another study that compared immunity development in two adult cohorts with similar exposures to schistosomes (Black*et al*. 2010b).

This current study is based on findings reported by our group that repeated treatment after reinfection can be associated with an increase in schistosome-specific IgE and $CD23^+$ B cells in children(Black *et al*. 2010a). These two responses have previously been reported to be associated with resistance to reinfection with *S. mansoni*(Mwinzi *et al*. 2009; Webster *et al*. 1997; Caldas *et al*. 2000).Other studies on reinfection rates after treatment indicate that adults in general have lower intensities of reinfection than children, even in situations where the level of exposure to the parasite is higher among adults than children(Kabatereine *et al*. 1999). It is also evident from epidemiological studies in areas of endemicity that adults suffer fewer infections

compared to children, accounting for the level of exposure in the two groups. This implies that resistance to infection develops with age, manifesting near puberty years(Kabatereine *et al*. 1999).

2.5 Immune correlates of protection

The mechanism by which treatment may cause an increase in resistance to reinfection is not fully understood. Before treatment, schistosome worms live in the blood for many years, ranging from an average of three to five years, and some up to 40 years, all this time releasing antigens(Warren *et al*. 1974; Chabasse *et al*. 1985; van Dam *et al*. 1996). Some antigens are however concealed from the immune system until the death of the worm either naturally or by a drug like PZQ. The worm's outer tegument is disrupted by PZQ thus exposing the underlying concealed antigens to the immune system consequently boosting anti-worm responses(Shaw and Erasmus 1987). This is also the possible explanation for the immunity to reinfection seen in some adults and older children who are exposed to naturally dying worms, having harboured infections longer than younger children. Several immune correlates of resistance have been described. A past study in a Kenyan population reported increased post-treatment worm-IgE which correlated with reinfection immunity and has subsequently been considered one of the correlates of protection against re-infection(Webster *et al*. 1997). This implies a response involving larval killing effected by eosinophils. Peripheral blood eosinophilia has been reported as another correlate of immunity(Hagan *et al*. 1985). Other studies have reported production of IL-5 and IL-4 by PBMCs *in vitro* in response to parasite antigens as other correlates of

resistance(Roberts *et al*. 1993) as well as elevated CD23+ B lymphocytes (Mwinzi *et al*. 2009).

There are increasing concerns about the possible loss of efficacy of PZQ following the scaling up of MDA in shistosomiasis endemic areas. The continued reliance on a single drug in the rolling out of MDA indeed raises prospects of development of resistance and tolerance. These concerns have been heightened by reports of reduced efficacy of PZQ in field studies in some settings and induction of resistance in laboratory-maintained parasite isolates. There has however not been any report of definitive evidence of clinically relevant resistance to the drug(Doenhoff *et al*. 1999). Black and colleagues have also reported findings on PZQ efficacy following 12.5 years of intense repeated use of the drug in a cohort of occupationally exposed adults. There was no evidence of a pattern of cure failures associated with development of resistance to the drug in this group (Black *et al*. 2009)

2.6 Problem statement

Standard age prevalence curves for schistosomiasis in endemic areas which are based on egg excretion show a gradual decline of prevalence over years while infection intensity sharply declines following a peak for both in the mid teen years (Kabatereine *et al*. 1999). This decline in prevalence and infection intensity has been attributed to development of acquired protective immunity to new infections. This is thought to occur following increased exposure to worm antigens released after the natural death of worms or as result of chemotherapy. An earlier study by our group involving adult car washers who are occupationally exposed to cercaria-containing water reported that some individuals developed increased resistance to re-infection following multiple (7-9) cycles of treatments, re-infections and re-treatment with PZQ(Karanja *et al*.

2002). A more recent study also by our group involving 8-10 year old school children reported an increase in schistosome-specific IgE and $CD23^+$ B cells after repeated treatment following reinfection with *S. mansoni* (Black*et al*. 2010a).It is however not known at what age and after how many rounds of treatment following reinfection these immunological changes occur. This is very important especially now when many countries including Kenya are soon to roll out MDA or are already implementing it in areas where schistosomiasis is endemic. This study seeks to determine the age at which children develop these immune correlates of protection against reinfection and if this age is altered by annual MDA.

2.7 Expected application of results

Findings from this study will be of great relevance to the ministries of health/public health in countries where the disease is endemic. This will in particular inform the decision on the best way to implement the World Health Assembly resolution 54.19(WHA 54.19) that calls for the regular treatment of all school age children at risk of contracting schistosomiasis. It will help to determine if there are additional benefits of chemotherapy. It may particularly lend evidence to the postulation that chemotherapy effects immunological changes to the normal host-parasite relationship that may lead to increased resistance to re-infection and elevated immunoregulatory mechanisms that control morbidity upon subsequent re-infections.

2.8 Research questions

i. What are the age-specific differences in anti-schistosomeimmune responses in 6-17 year old school children?

ii. At what age do immune correlates of protection against reinfection of schistosomiasis increase in children increase?

iii. What is the effect of one round of annual mass drug administration on immune correlates of protection in school children?

2.9 Null hypotheses

i. There are no age-specific differences in immune correlates of protection against reinfection of schistosomiasis in children before the start of annual mass drug administration.

ii. There is no effect of one round of annual mass drug administration on immune correlates of protection in children.

2.10 Objectives

2.11 General objective

To determine age-specific differences in immune correlates of protection of school children infected with schistosomiasis and the effect of treatment with praziquantel on the immune responses.

2.11.1 Specific objectives

i. To determine age-specific differences in anti-schistosome antibody isotype levels, cytokines and the proportion of $CD23^+$ B cells in school children with schistosomiasis

ii. To determine differences in levels of immune correlates of protection against reinfection of *S. mansoni* in school children and the age at which these immune correlates of protection begins to increase.

iii. To determine the effect of one round of annual mass drug administration on immune correlates of protection in children.

CHAPTER THREE:GENERALMATERIALS AND METHODS

3.1 Study area and subjects

This study was conducted in western Kenya, in primary and secondary schoolsin Asembo region of Rarieda sub-county in Siaya County near Lake Victoria (Fig 3.1).

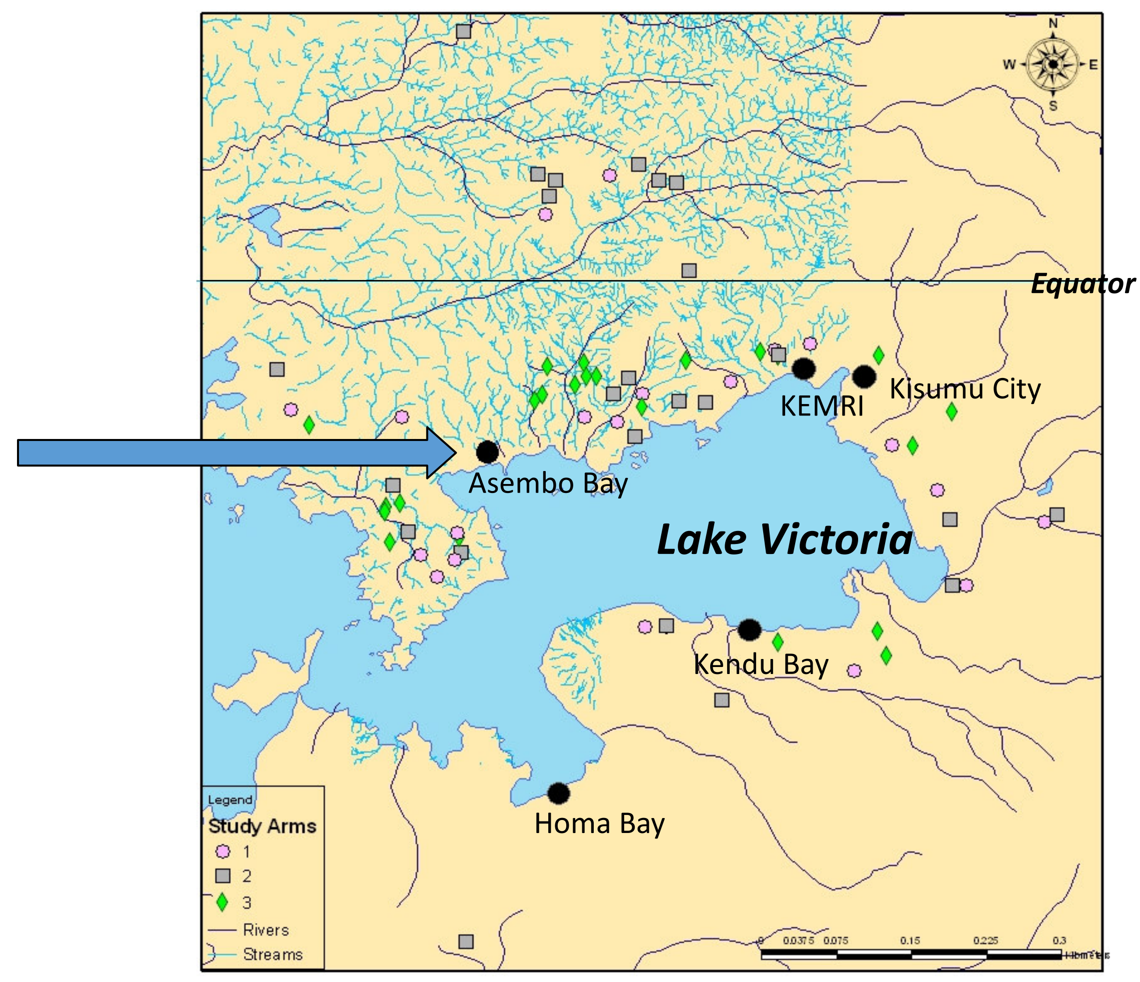


Figure 3.1: Map showing location of Asembo area (shown by arrow). Courtesy of the Schistosomiasis consortium for operational research and evaluation (SCORE) project.

This area is highly endemic for schistosomiasis mansoni, with a schistosomiasis prevalence of >50% (Handzel et al. 2003). The study subjects were school children, both male and female between the ages of 6 to 17 years and attending primary and secondary schools in Asembo Division in Rarieda District.Samples were transported to the NTD laboratories at the Kenya Medical Research Institute/Centers for Disease Control and Prevention's(KEMRI/CDCs),Centre for Global Health Research (CGHR), Kisian, Kisumu. It was in these laboratories that parasitological and immunological assays were performed.

3.2 Study design

This was a cross-sectional study that involved recruitment of 6 to 17 year old schistosomiasis mansoni positive school childrenfor establishment of immune responseprofiles. It also determined the age at which immune correlates of resistance to infection first increase. The children were treated with PZQ at baseline and after one year. In addition, one age group (7-13 years of age) wasexamined after one year. This was to determine ifone round of MDA alters the development of immune responses that correlate with resistance to reinfection.

3.2.1 Inclusion criteria

Subjects enrolled were both male and female who were 6to 17 years of age at the beginning of the study. They were attending primary and secondary schools in a village near Lake Victoria shoreline. They were egg-positive for schistosomiasis

mansoni upon diagnosis by stool examination. They were also willing to provide their assentand participate in the study, and informed consent had to be given by the guardian.

3.2.2 Exclusion criteria

Persons were excluded from the study if they had haemoglobin values below 8g/dl and were evidently ill according to the assessment of the clinician. Those unwilling to participate in the study, those not residing in the area, those who were outside the 6 to 17 year age bracket and those whose parents did not consent were also excluded from the study.

3.2.3 Sample size

Based on schistosomiasis prevalence rate of 65%(Handzel *et al*. 2003) from our eligibility study in the two primary and two secondary schools selected for this study, minimum sample sizewas determined according to the statistical formula:

$$N = \left(\frac{Z^2\, P(1-P)}{D^2}\right)$$

Where:

N=minimum sample size required

Z=1.96 standard error

P= postulated prevalence rate of 75% (0.75)

D=0.05=the inverse of 95% confidence limit

Substituting,

N= $\left(\frac{1.96^2\, x0.75(1-0.75)}{0.05^2}\right)$

=288

This is the number recruited at baseline, with a minimum of 20 participants coming from each class of primary school or form in secondary school. This being a repeated cross-sectional study, 99 participants of ages 7-13 years were recruited from the primary schools at follow-up after 1 year. Follow-up participants were recruited from class 2-3 and must have undergone MDA at baseline.

3.3 Specimen collection and handling

The specimens from this study were blood and stool specimens. Blood collection was done aseptically and specimens handled using universal precautions. Eight millilitres of blood were collected by venipuncture into heparinised vacutainer tubes. The tubes were then labelled using printed bar-coded stickers with the assigned subject number and brought from the field to the laboratory in styrofoam containers to maintain constant temperature.

3.4 Laboratory procedures

3.4.1 Parasitology

Stool samples were examined for helminth eggs by standard Kato-Katz smears involving 3 consecutive stool samples, two slides each(Katz *etal*. 1972). This technique diagnosed*S. mansoni*, *Ascaris lumbricoides*, and *Trichuris trichuria.*Malaria was diagnosed using Giemsa- stained blood smears made from finger-stick blood and examined by microscopy.

3.4.2 Whole blood culture

Whole blood culture was performed using 1.75 ml of heparinised blood diluted to a ratio of 1:5 in RPMI-1640 containing penicillin-streptomycin and L-glutamine. The blood was thencultured in 24-well microtitre plates, 1.5 ml per culture in the presence

of the mitogen phytohemagglutinin (PHA), schistosome antigens, that is soluble worm antigen preparation (SWAP) and soluble egg antigen (SEA), or RPMI alone. After 5 days of culture (3 days for PHA plates), the supernatant fluids were separately harvested and stored frozen (-20 °C) until assayed for cytokine levels (Appendix II).

3.4.3 Cell surface staining and flow cytometry

Four hundred microlitres of heparinised whole blood was used for cell surface staining. Four flow cytometry tubes were set up as follows: tube1, unstained as negative control, tube 2 anti-CD19 FITC, tube 3, anti-CD23 APC and tube 4, both anti-CD19 FITC and anti-CD23 APC conjugated antibodies. Data acquisition was done on a dual laser FACSCalibur flow cytometer (BD Biosciences, San Jose, CA). Data analysis wasdone as previously reported by Mwinzi and group (Mwinzi *et al.* 2009) and using FlowJo software version10.0.8 (Tree Star Inc. Oregon).Lymphocyte population was defined by non-specific fluorescence from the forward (FSC) and side scatter (SSC) as parameters of cell size and granularity, respectively. Gating for lymphocytes is shown Fig. 3.2 A. Gating to separate CD23+ from CD23- B cells was based on fluorescence

minus one (FMO) stained samples (tube 2 with anti-CD19 FITC for FL1 and without anti-CD23 APC for FL4) (Fig. 3.2 B). Data presented is the proportion of CD19+ B cells expressing CD23 (Fig. 3.2 C).

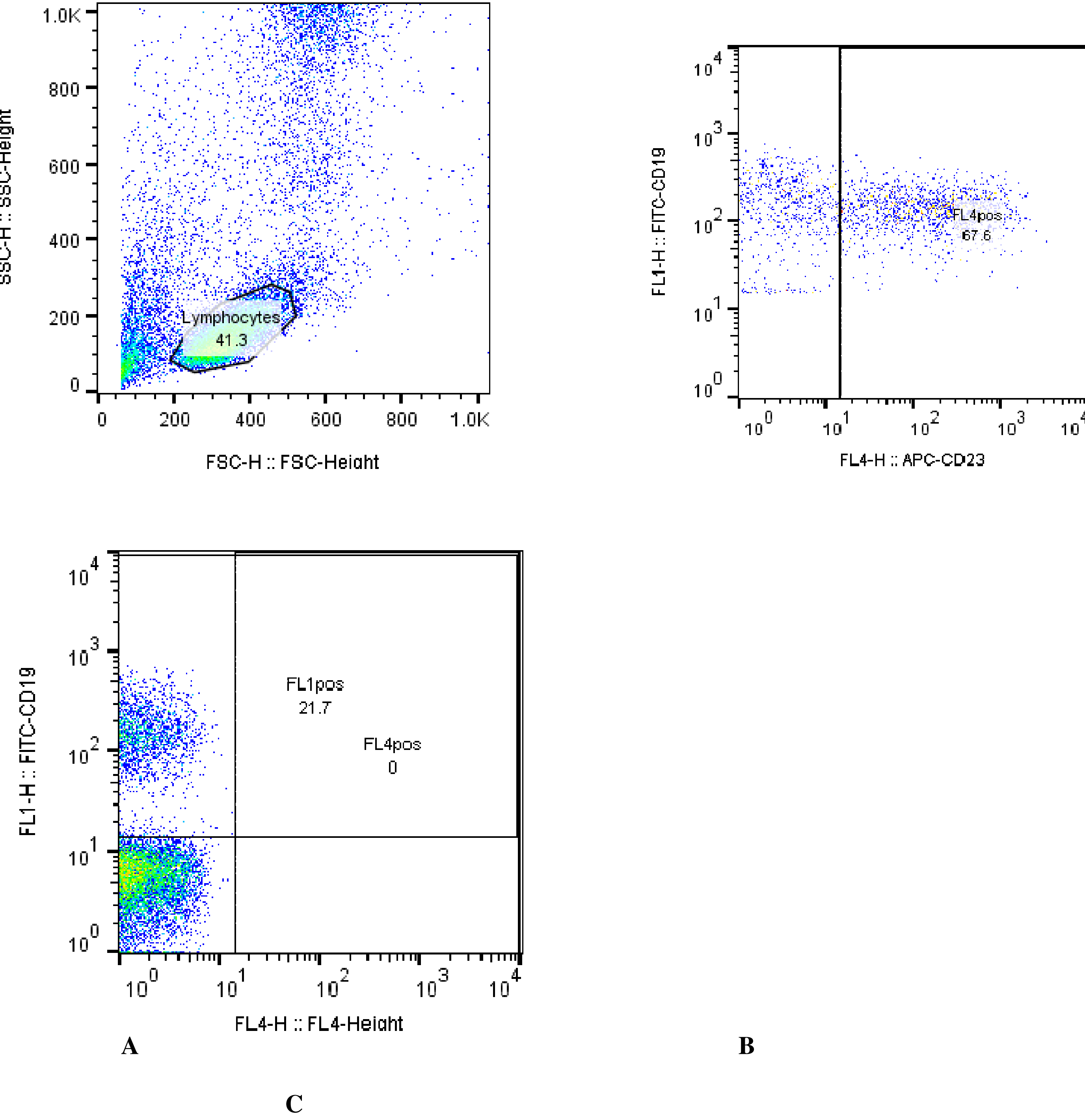


Figure 3.2: Flow cytometry data analysis density graphs. Graph A represents flow cytometry analysis of whole blood showing lymphocyte gate based on forward scatter (FSC) and side scatter (SSC) and used for all subsequent analyses. Cells were stained with anti-CD19 fluorescein isothiocyanate (FITC) and anti-CD23 allophycocyanin (APC). B shows gating for CD23+ B cells established based on fluorescence minus one (FMO) "no CD23-APC" controls and C shows the sub-set of B cells expressing CD23. FL-fluorescence parameter.

3.4.4 Cytokine ELISA

Assays for cytokines were performed by ELISA using commercial cytokine-specific monoclonal antibody pairs. The cytokines assayed from the frozen culture supernatant fluids wereInterleukin (IL)-5, IL-10, IL-13andIFN-γ.(Appendix II). Briefly, ninety six-well ELISA plates were coated with 100 µl of a given monoclonal capture antibody diluted in phosphate buffered saline (PBS) and incubated overnight at room temperature. They were then washed thrice (ELx405 Microplate washer, Biotek) with 0.05% Tween 20 (vol/vol), and blocked with PBS plus 10% bovine serum albumin (BSA) at 300 µl per well for 1 hour. The plates were then washed thrice and cytokine standards (R and D systems), serially diluted as per manufacturer's directions in respective reagent diluents. Samples diluted in RPMI at ratio of 1:1were added at 100 µl per well followed by 2 hour incubation at room temperature.

The plates were then washed thrice with the same wash buffer and working concentration of the appropriate monoclonal antibody (mab) was added at 100 µl per well for another 2 hour incubation at room temperature. This was followed by another three times wash before addition of streptavidin-horseradish peroxidise conjugate (R and D Systems) diluted in respective reagent diluents and added at 100 µl per well. This was followed by 20 minute incubation at room temperature before another round of 3 washes. Tetramethylbenzidine peroxidise substrate (TMB) was added at 100 µl per well and colour developed at room temperature. After development of colour (about 15 minutes), stop solution (1N sulphuric acid) was added at 50 µl per well. The optical density of each well correlated to cytokine quantity and was determined immediately using an automated microplate reader at 450 nm. IFN-γ reagent diluent

was 1xPBS+10% BSA while reagent diluent for IL-5, IL-10 and IL13was Trizma base+1% tween20 +1% BSA(Appendix II).

3.4.5 Serologic assays for schistosome-specific total IgG, IgE and IgG4 antibodies

The remaining heparinised whole blood (~5.75 ml) was centrifuged to yield approximately 3 ml of plasma, which was stored in aliquots frozen at -20^0 C until antibody assayswere performed. The assays were performed to detect anti-schistosome SEA and SWAP-specific total IgG, IgG4, and IgEand Tegument Allergen-like antigen (TAL) 1, TAL 2 or TAL 5)-specific IgE and IgG4 by ELISA.

3.4.6Data processing and analysis

Data was entered and cleanedin excel spreadsheets (MS® Office) and exported into GraphPad Prism software (GraphPad Inc., California, USA), version 5 for analysesCytokine, antibody and proportions of CD23+ B cells at baseline among age groups were comparedusing non-parametric Kruskal Wallis test for non-parametric data, with Dunn’s multiple comparisons test for post-hoc analyses. Parameric data was analysed using ANOVA for parametric data with Tukey’smultiple comparison post-ANOVA test. Paired responses between follow-ups were compared using Mann Whitney U test. All the tests were two-tailed at α-value of 5% ($P<0.05$) for statistical inferences.

3.5 Ethical considerations

The rights, safety and well-being of study subjects werefully safeguarded as outlined by Good Clinical Practice (GCP). Informed consent process was followed and utmost measures taken to protect the privacy and confidentiality of information collected from participants. This included random assignment of numbers to participants which were used in all records instead of names. The study commenced only after review

and approval by the Ethical Review Committee of the Kenya Medical Research Institute (Appendix II). In addition, the study was approved by the Institutional Review Boards of both the Centers for Disease Control and Prevention (CDC) and the University of Georgia, USA.

3.5.1 Informed consent process

Permission was sought from the district education office for the then Rarieda district (now sub-county) and head teachers of participating schools. Details of the study, rights, obligations, responsibilities, risks and benefits were discussed with parents/guardians and their children and informed consent obtained before enrolment. Both the parents/guardians and their children were asked to sign the consent and assent forms, respectively,before witnesses if they agreed to participate. The forms were translated into the local language, Dholuo, for parents who did not understand English (Appendix I).

CHAPTER FOUR: PRE-TREATMENT ANTI-SCHISTOSOME IMMUNOLOGICAL PROFILE IN A CROSS SECTION OF SCHOOL CHILDREN

4.1Introduction

Children in schistosomiasis endemic areas bear a greater burden of the disease than adults especially in terms of higher prevalence, worm burden and disease-associated morbidities.The disease is of serious health concern in developing countries, particularly in sub-Saharan Africa, Caribbean and South America. Among parasitic diseases, it is second only to malaria in its impact on human health. Age-infection profiles from disease endemic areas have a typical peak in infection intensities and prevalence in early adolescence followed by a steady decline towards adulthood. This provides epidemiological evidence of a natural age-dependent partial development of immunity to the disease(Fulford *et al.* 1998; Kabatereine *et al.* 1999). Treatment alone has largely not been sufficient to halt transmission, in part, due to inadequate coverage of infected populations and the fact that individuals remain susceptible to re-infection after treatment.

Susceptibility to schistosomiasis infection is linked to several factors, including genetic predisposition, host immune responses and environmental and behavioural factors (Oliveira *et al.* 2012). Many studies have been done to elucidate host anti-schistosome immune responses in both human and animal models. Human studies have however been mostly limited to epidemiological and *in vitro* experiments. Differences in schistosome-specific immune responses have been attributed to age, stage of disease and duration of infection (Black *et al.* 2009). The decline in infection intensities observed beginning in the late teenage years mentioned above is hypothesised to be a result of partial acquisition of protective immunity in this age group. The precise mechanisms by which human hosts resist schistosome infection

are not well understood. Studies have however shown that resistance to re-infection can be naturally acquired or induced following multiple rounds of exposure, treatment and re-infection (Corrêa-Oliveira *et al*. 1998; Karanja *et al*. 2002).

Several schistosome-specific immune correlates of protection have been identified. These are associated with T_H2 immune responses, high levels of IgE, recruitment and activation of eosinophils and mast cells (Oliveira *et al*. 2012; Black*et al*. 2010a). High levels of anti- SWAP IgE have been associated with resistance to re-infection in both adults and children, while high levels of IgG4 SEA and SWAP have been associated with susceptibility to re-infection (Figueiredo *et al*. 2012; Vereecken *et al*. 2007). IgG4 and IgE possibly display similar recognition specifities, leading to postulation that IgG4 blocks the protective effector functions of IgE (Hagan *et al*. 1991; Demeure *et al*. 1993).

Anti-*Schistosoma mansoni* tegument-allergen-like protein(smTAL)-1 IgE is another marker for immunity in schistosomiasis mansoni (Dunne *et al*. 1992). The parasite smTAL-1 antigen belongs to a family of well-characterized *S. mansoni* proteins denoted Sm21.7, Sm20.8 and Sm21.6 (Francis and Bickle 1992; Hoffmann and Strand 1997; Lopes *et al*. 2009). These proteins have a characteristic C-terminal region resembling dynein light chain (DLC)-1 domain and two N-terminal EF-hand motifs (Vichasri-Grams *et al*. 2006). The presence of EF-hand motifs gives these proteins close structural similarity to a common group of clinical allergens, the EF-hand allergens, hence the name, tegument-allergen-like (TAL) proteins *(*Fitzsimmons *et al*. 2012a).Alongside high anti-schistosome IgE, $CD23^+$ B cells in children have also been reported to increase after treatment(Black *et al*. 2010a). These two immunological markers have previously been reported to be associated with resistance to re-infection with *S. mansoni*(Mwinzi *et al*. 2009)

Cellular immune responses are also important in schistosomiasis infections, with strongest association with resistance to reinfection being increased production of T_H2 cytokines IL-5 and IL-4 in response to parasite antigens *in vitro*(Roberts *et al.* 1993; Ribeiro de Jesus *et al.* 2000; Walter *et al.* 2006). The regulatory cytokine IL-10 has been implicated in blockage of therapeutic-induced resistance development in an experimental model (Wilson *et al.* 2011).

Immunity to schistosome reinfection that seems to develop with age or exposure duration in older children or younger adults in schistosome-endemic areas is hypothesised to be a result of natural death of older worms. The lifespan of schistosome worms ranges from 5 up to 30 years(van Dam *et al.* 1996). The death of worms naturally or following treatment releases antigens that are otherwise concealed from the immune system. Treatment with PZQ disrupts the worm's outer tegumentthus exposing the underlying concealed antigens to the immune system consequently boosting anti-worm responses(Shaw and Erasmus 1987).

This study sought to profile anti-schistosome immune responses in a cross-section of school children before roll-out of mass drug administration with PZQ. From the profile, it should be possible to determine fairly well any age-specific acquisition of anti-schistosome protective immunity. To achieve this, anti-schistosome antibody and cytokine responses in 6 to 17 year old primary and secondary school children were assayed. Another correlate of immunity development examined in this study was the Proportion of CD23+ B lymphocytes of the study participants.

4.2 MATERIALS AND METHODS

4.2.1 Study area and subjects

Participants in this study were school children of ages 6-17 attending neighbouring primary and secondary schools in Asembo, an area near Lake Victoria, in Rarieda sub County of Siaya County in Western Kenya. A total of 288 participants were recruited into the study at baseline following parasitological assessment in the four schools to determine infection prevalence.This was part of a larger 5 year repeated cross-sectional study evaluating the effect of annual MDA on morbidity and immunological profiles in school children. Each participant turned in three stool samples for pre-treatment parasitological assessment using Kato-Katz on two slides per stool in the Neglected Tropical Diseases (NTD) parasitology laboratory at the Kenya Medical Research Institute's Centre for Global Health Research. Infection intensity was expressed in eggs per gram of faeces. Stool examination also included other soil transmitted helminths. About 8ml of venous blood was collected from the participants into heparin tubes and transported to the main immunology laboratory for assays and for malaria diagnosis.

4.2.2 Ethics statement

This study received ethical approval from the Ethical Review Committee of the Kenya Medical Research Institute. Details of the study, rights, obligations, responsibilities, risks and benefits were discussed with parents/guardians and their children and informed consent obtained before enrolment. Both the parents/guardians and their children were asked to sign the consent and assent forms, respectively,before witnesses if they agreed to participate. The forms were translated into the local language, Dholuo, for parents who did not understand English.

4.2.3 Immunological assays

4.2.3.1Whole blood phenotyping

Heparinised blood (400µl) was processed for staining using FITC conjugated anti-CD19 and APC conjugated anti-CD19 monoclonal antibodies as previously described (Black et al. 2010a). Data acquisition was done on a dual laser FACS Calibur flow cytometer and data analysed using FlowJo software version 10.0.8 (Tree Star Inc. Oregon). B cells were defined as CD19 positive. Cells positive for CD23 marker were gated based on fluorescence minus one (FMO) as previously reported here and in earlier study by Mwinzi and group(Mwinzi *et al*. 2009).

4.2.3.2Whole blood cultures

Whole blood culture was done using 1.75 ml of heparinised blood diluted to a ratio of 1:5 in RPMI-1640 containing penicillin-streptomycin and L-glutamine. The blood was thencultured in 24-well microtitre plates, 1.5 ml per culture in the presence of the mitogen phytohemagglutinin (PHA), schistosome antigens, that is soluble worm antigen preparation (SWAP) and soluble egg antigen (SEA), and RPMI alone. After 5 days of culture (3 days for PHA plates), the supernatant fluids were separately harvested and stored frozen (-20 °C) until assayed for cytokine levels

4.2.3.3 Antibody evaluation

The rest of the blood was centrifuged at 13300rpm to separate plasma, which was aliquoted into cryovials for storage at -20^{0}C until assayed for anti-schistosome antibodies by ELISA. Anti-SEA, anti-SWAP and anti-TAL IgE, IgG and IgG4 antibodies were determined using a standardised ELISA. Briefly, 96 well 2HB Immunlon microtitre plates were coated with antigens diluted in 0.1M sodium carbonate, pH 9.6. Coating antigen concentrations were as follows: 5ug/ml SWAP,

0.625ug/ml for anti IgG/IgG4 SEA, 2.5ug/ml for anti-SEA IgE, 2ug/ml for SmTAL-1 and 4ug/ml for both SmTAL-2and SmTAL-5. Coating incubation was for 2 hours at RT on titre plate shaker (Lab-Line Instruments Inc., Melrose Park, Illinois, USA), speed 1.2. Plasma samples were assayed in duplicate and standards in single wells. For IgE, plasma wasdiluted 1:20 and for IgG/IgG4 plasma was diluted 1:50 in 1xPBS+0.3% Tween 20+5% non-fat dry milk powder. Plasma sample incubation was at RT for 30 minutes on titre plate shaker speed 1.2. Secondary HRP conjugated antibodies used were: Mouse anti-human IgE-HRP (clone: B3102E8; Southern Biotech cat# 9160-05), Mouse anti-human IgG4-HRP (clone:HP6023;Southern Biotech cat# 9190-05) and Mouse anti-human IgG-HRP (clone:JDC-10; Southern Biotech cat# 9040-05). Conjugate antibody incubation was for 30 minutes at RT on titre plate shaker speed 1.2 Assays were developed using TMB substrate (Sigma) and absorbance read by Emax precision microplate reader (Molecular Devices Inc., California, USA). Primary antibody concentrations were extrapolated from standard curves based on apool of SWAP-IgE positive sera from previous studies

4.2.3.4 Cytokine assays

Schistosome-specific cytokines were assayed by ELISA on plasma supernatants harvested from whole blood cultures. The cytokines assayed were IL-5, IL-10, IL-13 and IFN-γ using DuoSet ELISA development system (R&D Systems, Inc., Minneapolis, Minnesota, USA) according to manufacturer’s protocols.

4.3 RESULTS

4.3.1 Baseline characteristics

A total of 288*S. mansoni* infected school children were recruited at baseline in this study from 4 neighbouring primary and secondary schools, 2 each. Participant ages ranged from 6 years to 17 years and 167 of them (58.0%) were girls (table 4.1). About 25-30 participants were recruited from each of the 12 classes in primary and secondary school. Males participants had significantly higher parasite burden (median, 112 eggs per gram of faeces (epg); Interquartile range, 196 epg; P=0.009) compared to females (median, 60 epg; IQR, 133 epg). After stratification of participants into 4 age categories; ≤8 years, n=19; 9-11 years, n=80; 12-14 years, n=90; and 15-17 years, n=99, there was significant difference in infection intensity across the age categories (P=0.044), with the post-test showing the 12-14 year olds having significantly higher median epg (130, $P<0.05$) compared to 15-17 year olds (median, 60 epg). (Table 4.1).

Table 4.1: Characteristics of study participants at baseline

Characteristics	Number (%)	Parasite burden (epg) median (IQR)	*P* value
Gender			
Male	121 (42.0)	112 (196)	**0.009**
Female	167 (58.0)	60 (133)	
Age category			
≤8 years	19 (6.6)	64 (136)	**0.044**
9-11 years	80 (27.8)	62 (150)	
12-14 years	90 (31.3)	130 (190)*	
15-17 years	99 (34.3)	60 (113)	

Data presented are parasite burden as estimated by eggs per gram of faeces (epg) by Kato-Katz technique. IQR-interquartile range. Female and male Median epgs were compared by Mann Whitney U test while epgs for the different age categories were compared by Kruskall Wallis test and Dunn's multiple comparison post-test. Significant *P* values in bold. *P<0.05.

4.3.2 Pre-treatment anti-schistosome antibody levels

4.3.2.1 Anti-SEA and anti-SWAP antibody levels

While most of the anti-SWAP and anti-SEA antibodies did not differ significantly across the different age-groups, anti-SEA IgE and total IgG were significantly different across the age-groups ($P<0.0001$). Subsequent post-hoc analysis showed that the 9-11 age group had higher anti-SEA IgE (median, 433.8; Interquartile range, IQR, 692.0 arbitrary units, AU) compared to the 15-17 year olds (median, 90.3; IQR 160.0 AU; $P<0.0001$). Similarly, anti-SEA IgE was higher in the 12-14 year olds (median, 268.1; IQR 497.9 AU) relative to 15-17 year olds (median, 90.3; IQR 497.9 AU; $P<0.0001$). The pattern for anti-SEA IgG was quite the opposite, with 9-11 year olds producing less IgG (median, 20.6; IQR 120.2 AU) than 15-17 year olds (median, 201.2; IQR 488.6 AU; $P<0.0001$). Moreover, 12-14 year olds had less antibody (median, 54.9; IQR 177.1 AU) than 15-17 year olds (median, 201.2; IQR 488.6 AU; $P<0.0001$). (Figure 4.1).

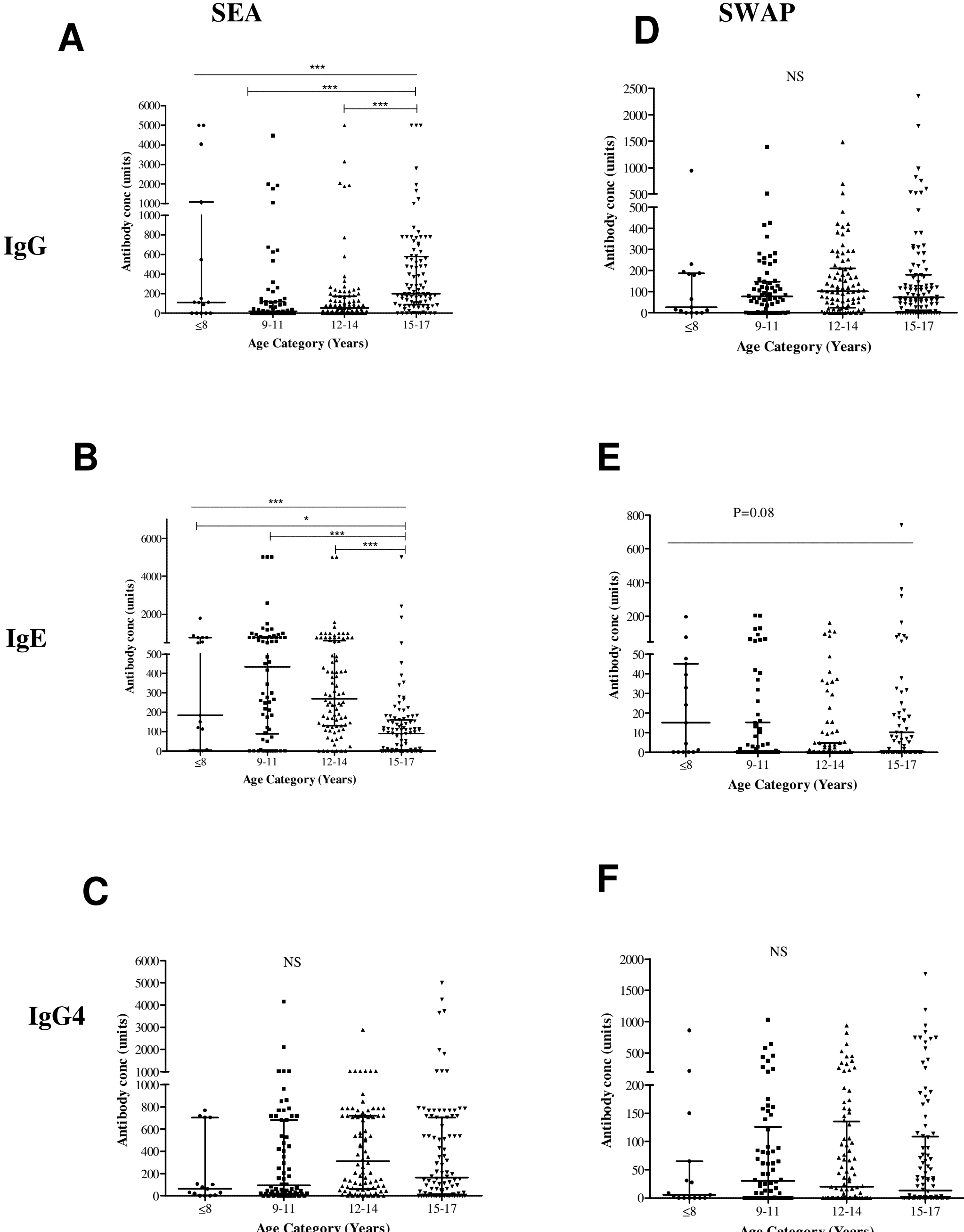


Figure 4.1: Plasma anti-soluble egg antigen (SEA) and anti-soluble worm antigen preparation (SWAP) antibody levels. Scatter plots showing antibody levels (arbitrary units, AU) in school children categorised into 5 age groups, 8 years and below (n=15), 9-11 years (n=66), 12-14 years (n=88) and 15-17 years (n=96). The left panel depicts the anti-SEA results by isotype: Immunoglobulin (Ig)G (A); IgE (B); IgG4 (C) and the right panel shows anti-SWAP results by isotype: IgG (D); IgE (E) and IgG4 (F). Data are presented as dot plots with line at median and whiskers at 75th and 25th percentile.Data analysis by Kruskall Wallis and post-test by Dunn's multiple comparison test. *P<0.05, *P<0.001 and ***P<0.0001.

4.3.2.2 Anti-TAL antibody levels

Anti-SmTal 1, SmTAL 2 and SmTAL 5 IgE and IgG4 were assayed in this study. Overall, none of the anti-smTAL antibody responses were statistically different across the age groups. Anti-SmTAL 1 IgE was nearly significantly (*P*=0.076) different across the age groups (Fig 4.2A) with mean antibody levels being higher in older children (mean, 7.86; SEM, 3.356 AU and mean, 7.396; SEM 2.191 AU in the 12-14 year and 15-17 year olds respectively) than in younger children (mean 3.88; SEM 1.997 AU in the 9-11 year olds). The rest of the antibodies assayed did not differ significantly with age. These are anti-TAL 1 IgG4, (*P*=0.553; Fig. 4.2B), anti-TAL 2 IgE (*P*=0.952; Fig. 4.2C), and both IgE and IgG4 against SmTAL 5 (*P*=0.170 and *P*=0.611 respectively;Fig. 4.2E and F).

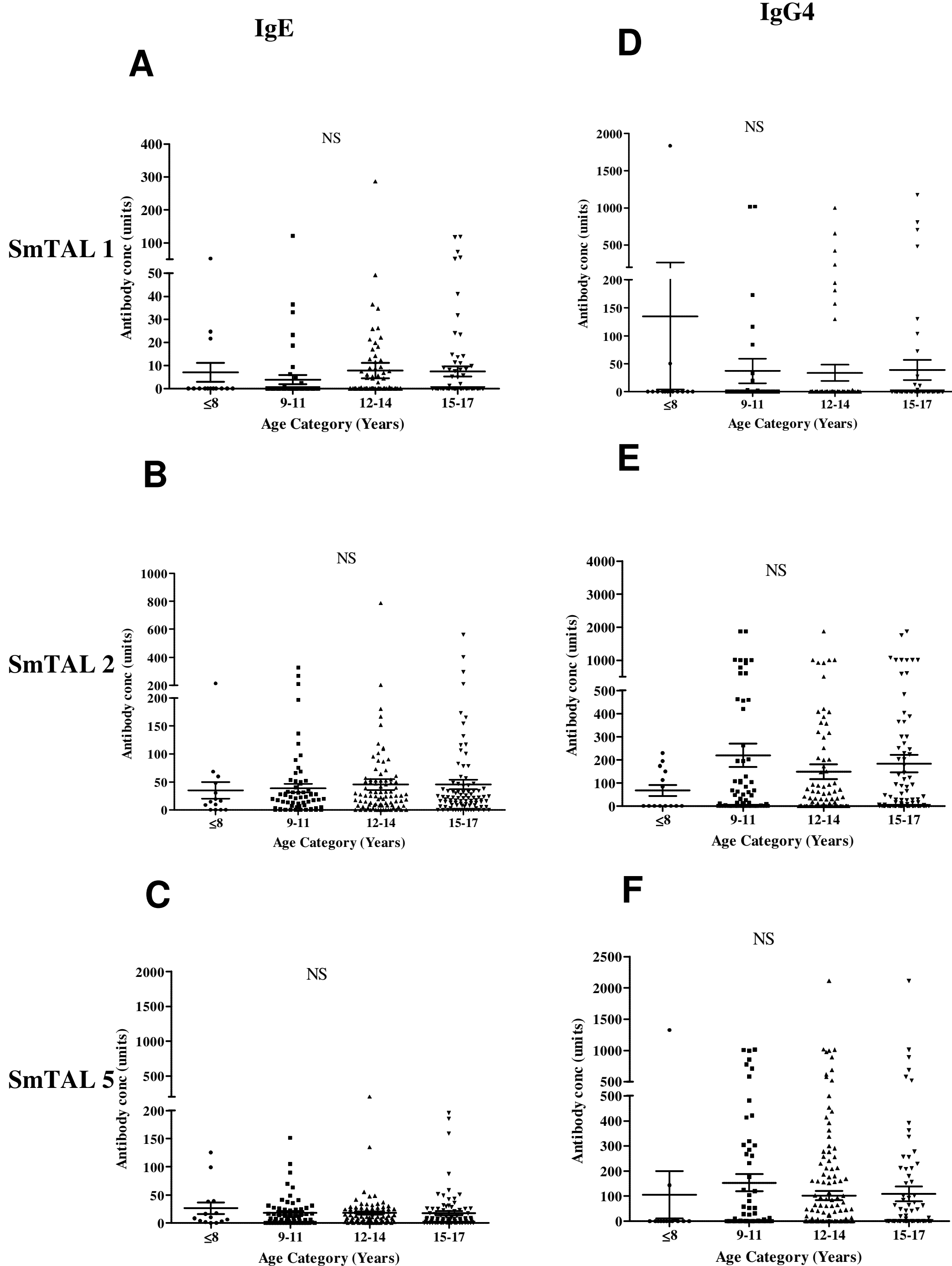


Figure 4.2: Plasma anti-SmTAL 1, SmTAL 2 and SmTAL 5 antibody levels. Scatter plots showing antibody levels (arbitrary units, AU) in school children categorised into 5 age groups, 8 years and below (n=15), 9-11 years (n=66), 12-14 years (n=88) and 15-17 years (n=96). Antibody isotypes shown in the left panel are Immunoglobulin (Ig)E anti-Schistosoma mansoni tegument allergen-like (SmTAL) 1(A), SmTAL 2 (B) and SmTAL 5 (C) and on the right panel IgG4 anti-SmTAL I (D), SmTAL 2 (E) and SmTAL 5 (F). Data presented as dot plots with line at median and whiskers at 75th and 25th percentiles.Data analysis by Kruskall Wallis and post-test by Dunn's multiple comparison test. NS-*P* value not significant

4.3.2.3 CD23+ B cell proportions

Pre-treatment CD19+/CD23+ B cell proportions did not differ significantly across the age groups (Fig 4.3). There was however a general marginal increase in this B cell proportion from the lowest mean percentage (63.3; SEM, 2.9) in the 8 year old and below group and mean percentages peaking in the 12-14 year old group (68.5; SEM, 1.0) before the means decreased slightly in the 15-17 year old group (67.64; SEM, 1.107).

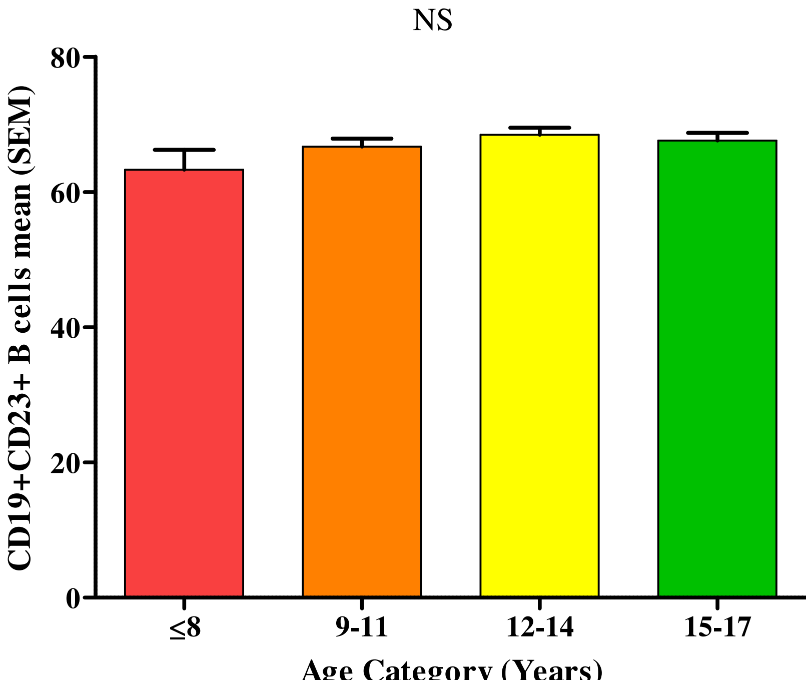
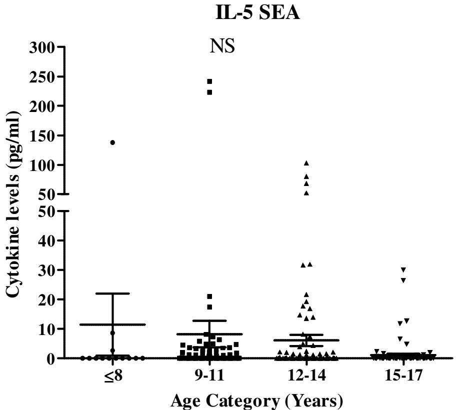
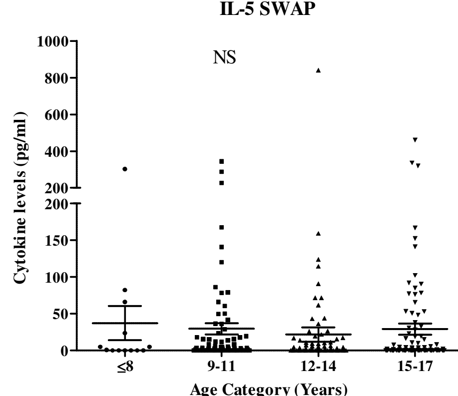


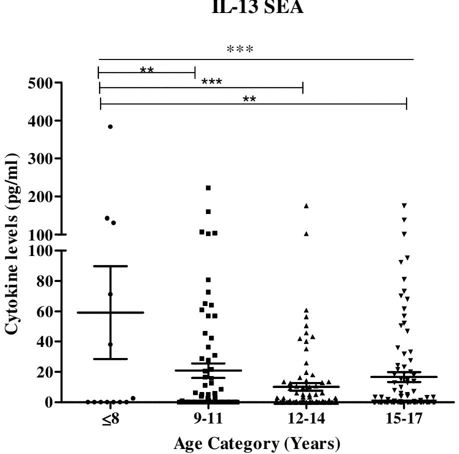
Figure 4.3: Percentage of CD19+ CD23+ B cells.Bar graph with barsshowing mean percentages and standard error of the mean (error bars) in school children categorised into 4 age groups, 8 years and below (n=15), 9-11 years (n=66), 12-14 years (n=88) and 15-17 years (n=96). Statistical analyses by ANOVA. CD19+CD23+ B cell percentages not significantly (NS) different among the age groups (P>0.05).

4.3.2.4 Cellular immune responses

There were nodifferences in the ability of either SEA or SWAP to stimulate whole blood cultures from the different age groups to produce IL-5, IL-10 or IFN-γ(Fig. 4.4 and Fig. 4.5). However, SEA and SWAP stimulation of IL-13 production differed statistically across the age groups (P=0.0001 and 0.0003, respectively) (Fig.4.5). Following Tukey's multiple comparison test on SEA-specific IL-13 means, the 8 year old and below group had higher mean (59.1; SEM, 30.5) than the 12-14 year olds (mean 10.1; SEM, 2.6; P<0.0001) and15-17 year olds (mean, 16.5; SEM, 3.3; P<0.001. Similar subsequent analyses also revealed that 8 year old and below group had higher mean SWAP-specific IL-13 (mean, 46.5; SEM, 17.9) than 12-14 year old group (mean, 13.5; SEM, 3.1; P<0.05). Additionally, the 9-11 year old group had also higher mean SWAP-specific IL-13 (mean, 29.3; SEM, 5.1) than 12-14 year old group (mean, 13.5; SEM, 3.1; P<0.05).







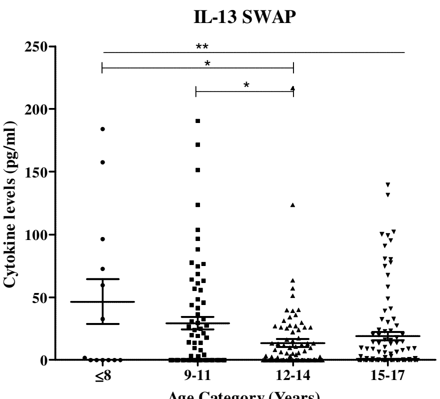
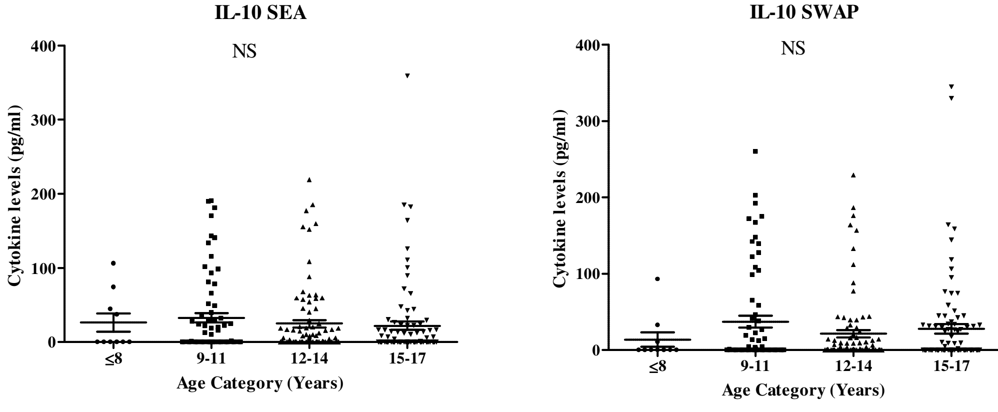


Figure 4.4: Plasma supernatant Interleukin (IL)-5 and IL-13 from whole blood cultured with soluble egg antigen (SEA), left panel or soluble worm antigen preparation (SWAP), right panel.Scatter plots showing cytokine levels in school children categorised into 5 age groups, 8 years and below (n=15), 9-11 years (n=66), 12-14 years (n=88) and 15-17 years (n=96). Data are presented as dot plots with line at mean and whiskers at standard error of the mean. Data analysis by ANOVA and post-anova test by Turkey's multiple comparison test. *P<0.05, **P<0.001 and ***P<0.0001



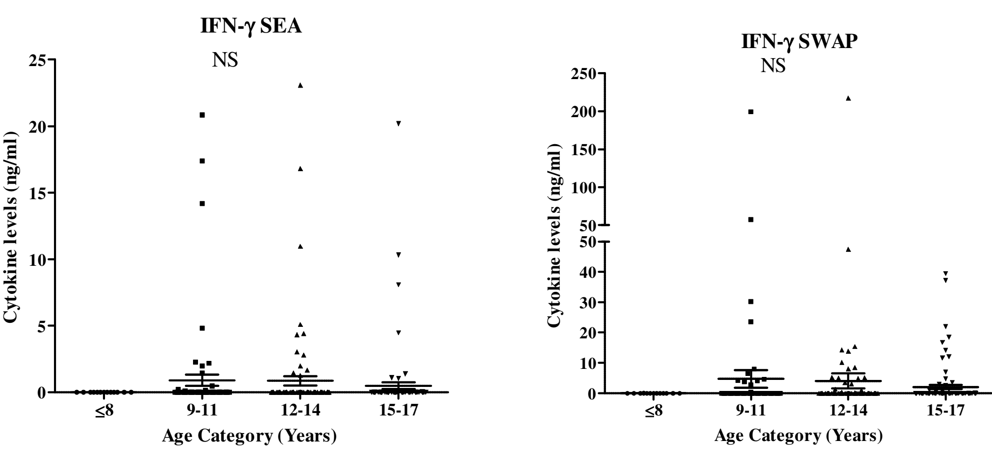


Figure 4.5: Plasma supernatant Interleukin (IL)-13 and Interferon (IFN)-γ from whole blood cultured with soluble egg antigen (SEA), left panel or soluble worm antigen preparation (SWAP), right panel. Scatter plots showing cytokine levels in school children categorised into 5 age groups, 8 years and below (n=15), 9-11 years (n=66), 12-14 years (n=88) and 15-17 years (n=96). Data are presented as dot plots with line at mean and whiskers at standard error of the mean. Data analysis by ANOVA and post-anova test by Turkey's multiple comparison test. *P<0.05, **P<0.001 and ***P<0.0001

4.4 DISCUSSION

This study sought to determine the age profile of *Schistosoma mansoni*-specific immune responses in schistosomiasis infected 7 to 17 year old school children living in an area endemic with the disease before treatment with praziquantel. This study coincided with the launch of national school-based deworming program targeting to control morbidity and prevalence of soil transmitted helminths and schistosomiasis through mass drug administration. Protective immunity against reinfection of schistosomiasis has been shown to develop with age among people living in endemic areas(Naus *et al*. 2003). Among the responses mostly associated with protection is IgE against adult antigens. We did not observe an age-associated increase in this response as has been reported in other studies (Naus *et al*. 2003; Webster *et al*. 1997). While children in this study are mostly from the same or neighbouring villages all within 5 kilometre radius of the lake, those of the same age group havebroadly divergent immune responses indicative of varying exposure histories to schistosome infection. Anti-SEA IgE levels on the other hand declined with age, a finding that is contrary to what has been reported previously, showing that levels of this antibody remain unchanged with age (Naus *et al*. 2003) or increased with age for the case *S. haematobium* in a Zimbabwean study (Ndhlovu *et al*. 1996). The hypothesis that egg antigens are constantly released after infection unlike adult antigens which are sequestered from the immune system until death of worms. Consequently, the constant exposure of egg antigens to the immune system would lead to tolerization and thus declining antibody levels with age as shown by results in this study. This understanding of the immunobiology of progressive *S. Mansoni* infection would fit with the observed data reported here.

Other antibody isotypes remained unchanged across the different age groups except anti-SEA total IgG which increased from the 15-17 year olds. This is in contrast to the negative correlation found between anti-SEA IgE and age in a Brazilian study (Negrão-Corrêa *et al*. 2014). This was however reported in a study that had a much wider age range of 5-68 years. We also examined anti-SEA and –SWAP IgG4 subtype and did not find any significant variation in this antibody with age. High levels of this antibody are associated with susceptibility to reinfection and tends to be elevated among children compared to adults (Hagan *et al*. 1991; Demeure *et al*. 1993).

Anti-smTAL 1 IgE responses somewhat increased with age in agreement with what has been reported previously (Fitzsimmons *et al*. 2012a). This recombinant antigen has been shown to be expressed primarily in the very early schistosomulae form and the adult stage of the parasite lifecycle (Fitzsimmons *et al*. 2006). It has been theorised that this antigen is among those concealed from the immune system until release at the natural death of the worms or following treatment. The trend where pre-treatment anti-smTAL-1 IgE increases with age fits with this hypothesis as older individuals who are presumed to have been infected earlier are exposed more to these antigens as their worms begin to die. This response is also associated with resistance to re-infection after treatment (Dunne *et al*. 1992). The two other recombinant antigens examined, smTAL-2 and smTAL-5 are expressed predominantly in the egg stage and cercariae and adult stages, respectively(Fitzsimmons *et al*. 2012b). Only anti-smTAL-2 IgG4 increased with age in contrast to findings in a study done among 7-16 year old children (De Moira *et al*. 2013).

Most of the cytokine responses to stimulation with schistosome antigens in vitro did not differ with age, with the exception of IL-13 which tended to decrease in the older

age groups.This cytokine has been implicated in morbidity during schistosomiasis infection, most notably with development of fibrosis(Oliveira *et al*. 2006). Interleukin 5, the other Th2 cytokine examined in this study correlates with protection against reinfection and SWAP-specific IgE (Walter *et al*. 2006). It is therefore not surprising that levels of this cytokine produced in response to SWAP did not show any age-associated pattern just as was observed with anti-SWAP IgE in this study.

CHAPTER FIVE: EFFECT OF TREATMENT WITH PRAZIQUANTEL ON SCHISTOSOME-SPECIFIC IMMUNE RESPONSES INA GROUP OF SCHOOL-AGE CHILDREN

5.1 Introduction

Schistosomiasis and other NTDs are increasingly receiving attention in the world today. This is following greater awareness of the extent of loss of quality of life among infected persons, majority of whom live in sub-Saharan Africa (SSA) and their contribution to poverty (Hotez and Kamath 2009). The World Health Organization's (WHO's) roadmap for NTD's set targets for the control, prevention, elimination and eradication of 17 NTD's including schistosomiasis (WHO 2012). This inspired key stakeholders and partners of WHO to make commitments to support the realization of these targets by making the London Declaration on Neglected Tropical Diseases(WHO 2013).

It is estimated that 93% of persons infected with schistosomiasis (192 million) live in SSA(King and Dangerfield-Cha 2008). About one third of schistosomiasis cases in this region are due to infection with *Schistosoma mansoni,* which is also estimated to result in about 130, 000 deaths annually (King and Dangerfield-Cha 2008). In Kenya, both *S. haematobium* and *S. mansoni* are endemic, with well established focal distribution. Up to 6 million people are at risk of infection in the country (Chitsulo *et al*. 2000). In the western part of the country, infection is predominantly by *S. mansoni*, with prevalence in school children increasing with proximity to Lake Victoria which is the main source of infection in the region (Handzel *et al*. 2003; Brooker *et al*. 2001).

The mainstay for preventive chemotherapy of schistosomiasis has been Praziquantel (PZQ) since 2006 (WHO 2006). The drug can be administered to all age groups as a

single dose of 40 mg kg^{-1} of body weight.It has been used in many countries in MDA both infected people and those at risk of infection. Mass drug administration involving PZQ was included for the first time in Kenya in a nationwide school-based deworming (SBD) campaign targeting major NTD's endemic in Kenya(WHO Kenya 2011; Ministry of Public Health and Sanitation Kenya 2011). So far, three rounds of annual SBD have been administered since launch of the program in 2012, with a target of reaching at least 75% of school- age children as recommended by the WHO (WHO 2001).The main benefit of preventive chemotherapy is the positive impact on morbidity, prevalence and in some cases transmission. The continued susceptibility of treated individuals however remains a major challenge to this control measure.

Epidemiological studies of human schistosomiasis infection in endemic areas reveal an age-dependent infection pattern and slow development of protective immunity. It has been proposed that dying worms are the main source of protective antigen, and given the long parasite life-span, exposure to these antigens is delayed (Woolhouse and Hagan 1999). Additionally, the induction of a protective response requires exposure to a certain threshold level of antigen (Mutapi et al. 2008). This process can be accelerated by manyrepeated treatments with PZQfollowing reinfectionswhich leads to the multiple release and exposure to the immune system of previously hidden parasite antigens (Fulford *et al*. 2006; Karanja *et al*. 2002).This protective resistance to reinfection however seldom leads to sterile immunity (Colley *et al*. 2014).

Several immunological correlates of protection against reinfection of schistosomiasis have been reported in previous studies, and are mainly T_H2 associated (Hagan *et al*. 1991; Roberts *et al*. 1993; Joseph *et al*. 2004). These include elevated parasite-specific IgE, anti-tegumental-allergen-like 1 (TAL-1) IgE, higher IgE and IgG4 ratio and increased number of eosinophils (Joseph *et al*. 2004; Hagan *et al*. 1991; Dunne *et*

al. 1992). Another correlate of the development of protection is the low affinity IgE receptor on B cells which is also reported to increase after repeated treatments over 2 years following reinfection in children (Mwinzi *et al*. 2009; Black *et al*. 2010a). Additionally, elevated IL-4 and IL-5 post-treatment have also been linked with resistance to reinfection while experimental mouse studies have shown that IL-10 blocks the development of post-treatment protective responses(Roberts *et al*. 1993; Joseph *et al*. 2004; Wilson *et al*. 2011).

Mass drug administration of praziquantel to school children in schistosomiasis endemic areas has become an integral component of the school-based deworming program in Kenya. This study seeks to examine the effect annual MDA has on schistosome-specific protective immune responses of children in their early teenage years. This is an age-group in which these responses are thought to naturally begin to increase according to age-infection curves of people living in schistosomiasis-endemic areas. The responses measured were schistosome-specific IgE, IgG4, total IgG, CD23+ B cell levels and schistosome-specific IL-5, IL-10, IL-13 and IFN-γ.

5.2 MATERIALS AND METHODS

5.2.1 Study area and subjects

Study participants were recruited from two neighbouring primary schools in Asembo area of Rarieda subcounty, Siaya County in western Kenya in a repeated cross-sectional study. They were a total of 262 participants, both male and female, attending class 2, 3 and 4 (ages 7-13 years) from two primary schools, with 163 out of 288 from the baseline group who were all schistosome egg positive. The other 99 were recruited 1 year after later and had all received praziquantel treatment in the previous year. Samples were collected in school and taken to the Kenya Medical Research Institute's

(KEMRI's) Centre for Global Health Research (CGHR), Kisian, Kisumu for storage and analysis.

5.2.2 Study design

This was a repeated cross sectional study in which classes 2 to 4 primary school children of ages7-13 whowere schistosomiasis infected at baseline were recruited. Participants selected for the study in the following year were those who hadreceived MDA involving PZQin the previous yearfor determination of the effect of treatment on immune correlates of protection against re-infection of schistosomiasis.

5.2.2.1 Inclusion criteria

Both male and female subjects in classes 2 to 4 were in this included in this study. They were attending two primaryschools in villages nearthe Lake Victoria shoreline. They received 1 round of PZQ and their immune responses studied 1 year later. They were also willing to provide their assentand participate in the study, and informed consent had to be given by the guardian.

5.2.2.2 Exclusion criteria

Participants with haemoglobin values below 8g/dl and were evidently ill according to the assessment of the clinician were excluded from the study. Additionally, those who did not give their assent to participate in the study, those not living in the area, those who were outside class 2, 3 and 4, those who did not receive PZQ treatment the previous year and those whose parents did not consent were also excluded from the study.

5.3 Specimen collection and handling

The specimens collected for this study were venous blood and stool. About 10 ml of blood was collected by venipuncture done by a qualified phlebotomist into heparinised vacutainer tubes. The tubes were labelled using printed bar-coded stickerseach with a unique subject number and transported to the laboratory in styrofoam containers to keep temperatureconstant. Stool samples were collected into stool cups and taken to the laboratory for diagnostic procedures.

5.4 Laboratory procedures

5.4.2 Whole blood culture

Part of the heparinised blood was diluted to ratio of 1:5 in RPMI-1640 containing penicillin-streptomycin and L-glutamine. This was then used to set up whole blood cultures in 24-well culture plates, using 1.5 ml per culture with each of the following stimulants: phytohemagglutinin (PHA), schistosome soluble worm antigen preparation (SWAP), soluble egg antigen (SEA), ormedia alone. The cultures were kept for 5 days (except for PHA which were harvested after 3 days)in a CO_2 incubator at 37^0C. Culture supernatant fluids were separately harvested into cryovials and stored frozen (-20 °C) until assayed later for cytokine levels (Appendix II).

5.4.3 Cell surface staining and flow cytometry

Immunophenotyping for CD23+ B cells was done as described earlier. Briefly, gating to separate CD23+ from CD23- B cells was based on fluorescence minus one (FMO) stained samples (in tube 2). Data acquisition was done on a dual laser FACSCalibur flow cytometer. Data analysis was done using FlowJo software version 10.0.8 (Tree Star Inc. Oregon).

5.4.4 Cytokine ELISA assay

Cytokine levels were determined by ELISA using commercial kits (R&D Systems Duo SET kits). The cytokines assayed from the frozen culture supernatant fluids after thawing were Interleukin (IL)-5, IL-10, IL-13 andIFN-γ. This was done as described in Appendix III and briefly as follows. Ninety six-well microtitre plates were coated with 100 µl of a given monoclonal capture antibody diluted in PBS, covered with parafilm and incubated overnight at room temperature (RT). Plates were then washed thrice (ELx405 Microplate washer, Biotek) with 0.05% Tween 20 (vol/vol), and blocked with PBS plus 10% BSA at 300 µl per well for 1 hour at RT. The plates were then washed as above thrice and cytokine standards (R and D systems), serially diluted as per manufacturer's directions in respective reagent diluents. Sample supernatant fluids were diluted in RPMI at ratio of 1:1wereand added at 100 µl per well to the plate followed by 2 hour incubation at RT.

Plates were then washed as before thrice with the same wash buffer and working concentration of secondary (detection) Mab added at 100 µl per well for another 2 hour incubation at RT. This was followed by another three times wash with was buffer before addition of streptavidin-horseradish peroxidise conjugate (R and D Systems) diluted in respective reagent diluents and added at 100 µl per well. This was followed by 20 minute incubation at RT before another round of 3 washes. Tetramethylbenzidine peroxidise substrate (TMB) was added at 100 µl per well and colour developed at RT. After development of colour (about 15 minutes), stop solution (1N sulphuric acid) was added at 50 µl per well. The optical density of each well correlated to cytokine quantity and was determined immediately using an automated microplate reader at 450 nm. IFN-γ reagent diluent was 1xPBS+10% BSA

while reagent diluent for IL-5, IL-10 and IL13 was Trizma base+1% tween20 +1% BSA(Appendix II).

5.4.5 Serologic assays for schistosome-specific antibodies of given isotypes

The rest of the heparinised whole blood (~5.75 ml) was centrifuged to yield about 3 ml of plasma, which was stored in aliquots frozen at -20^0 C until antibody assays were done. The assays were done to detect anti-schistosome SEA or SWAP-specific total IgG, IgG4, and IgE or Tegument Allergen-like antigens (TAL) (TAL-1, TAL-2 and TAL-5)-specific IgE and IgG4 by ELISA.

5.4.6 Data processing and analysis

Data was initially processed in excel spreadsheets (MS® Office) and exported into GraphPad Prism software (GraphPad Inc., Carlifornia, USA), version 5 for analyses. Cytokine, antibody and proportions of CD23+ B cells levels at follow-up were compared between *S. mansoni* infected and uninfected participants with baseline levels using non-parametric Kruskal Wallis test and Dunn's multiple comparison post-test. All the tests were two-tailed at α-value of 5% ($P<0.05$) for statistical inferences.

5.5 Ethical considerations

This study was carried out with utmost care to safeguard the rights, safety and well-being of study subjectsas outlined by Good Clinical Practice (GCP). Informed consent process was fully adhered to and utmost measures taken to protect the privacy and confidentiality of information collected from participants. This included random assignment of numbers to participants which were used in all records instead of

names. The study was protocol was reviewed by the Ethical Review Committee (ERC) of the Kenya Medical Research Institute and commenced only after necessary approvals had been granted. Both parents and or guardians and children were taken through details of the study, their rights, obligations, responsibilities, risks and benefits in order to make informed an informed decision on to participate in the study or not. Parents/guardians who consented that their children could take part in the study were asked to sign the consent forms and the children were for their assent before a witness. The forms were translated into the local Dholuo language for parents/guardians who did not understand English (Appendix I).

5.6. RESULTS

5.6.1 General characteristics

This repeated cross-sectional study involved a total of 262 participants, with 163 being part of a larger schistosomiasis infected cohort recruited at baseline and 99 recruited a year after treatment with praziquantel.The general characteristics of these 1 year post-treatment participants are given in table 5.1. Their ages ranged from 7 to 13 years. Male participants were 44.8% at baseline and 51% in the post-treatment group. The proportion male to female participants at baseline and 1 year post-treatment was not statistically different (*P*=0.322). Of the 99 participants in the post-treatment group, 62 (62.6%) of them were *S. mansoni*infected, a majority of them (56.5%) being male. In general, infection intensities were lower in the post-treatment, re-infected group (median, 28; IQR, 492 epg and median, 28; IQR, 388 epg) compared to pre-treatment levels (median, 112; IQR, 196 epg and median, 60; IQR, 133 epg) among males and females, respectively. Overall, baseline infection intensities were higher (median, 40; IQR, 176 epg) than post-treatment intensities

(median, 28; IQR, 68 epg; *P*<0.0001). Participants were stratified into three age categories: 7-8 years old, n=14 and n=30, 9-11 years old, n=80 and n=57 and 12-13 years old, n=69 and n=12 for pre- and post-treatment groups, respectively. The largest percentage of infected children 1 year post-treatment in each age group were in the 12-13 year old age group, at 83.3% although the total number of children in this age-group was the lowest (12 out 99). The proportions of infected children were significantly higher than un-infected ones in all age categories (*P*<0.0001)Additionally, the proportion of 12-13 year olds were more at baseline relative to the other age groups compared to 1 year post-treatment (*P*<0.0001). Conversely, the proportion of 7-8 year olds was higher in the 1 year post-treatment group (30.3%) compared to the baseline (8.6%, P<0.0001). Infection intensities were generally low after treatment, with the 9-11 year olds having the highest (median, 44; IQR, 158) followed by the 12-13 year olds (median, 24; IQR, 81 epg) and lowest among the 7-8 year olds (median, 17; IQR, 45 epg).

Table 5:1 Participant umbers and percentages and their infection intensities at baseline and 1 year post-treatment

Characteristics		Number (%)					
		Pre-treatment	**1 year post-treatment**				
			All	Sm +ve	Sm -ve	*P* Pre-treatment vs 1 year post treatment	*P* Sm+ vs Sm -ve SM
Gender							
Male		73 (44.8)	51 (51.5)	35 (68.5)	16 (31.4)	0.322	**<0.001**
Female		90 (55.2)	48 (48.5)	27 (56.3)	21 (43.8)		**<0.001**
Age Category	7-8 years	14 (8.6)	30 (30.3)	20 (66.7)	10 (33.3)	**<0.001**	**<0.0001**
	9-11 years	80 (49.1)	57 (57.6)	26 (45.6)	31 (54.4)	**0.0001**	**<0.0001**
	12-13 years	69 (42.3)	12 (12.1)	10 (83.3)	2 (16.7)	**<0.0001**	**<0.0001**

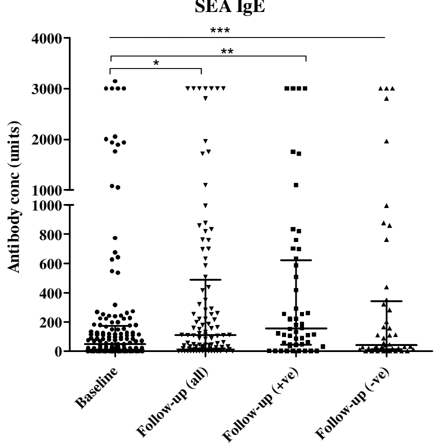
Infection burden (median epg)	40 (176)	28 (68)	28 (68)	-	P<**0.0001**	-

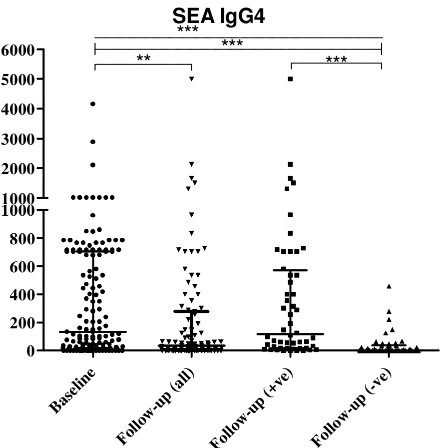
Data presented are numbers of participants with percentages in brackets and for infection intensity, median eggs per gram of faeces (epg) and interquartile rage in brackets. Sm+, Schistosoma mansoni egg positive by Kato Katz based on 3 stools, 2 slides each. Comparison of proportions by Chi square test. Significant *P* values in bold.

5.6.2 Anti-SEA and –SWAP antibody levels

Pre- and post-treatment anti-SEA and –SWAP IgE, total IgG and IgG4 responses were compared among children of the between 7 and 13 years.The 1 year post-treatment group was further split into the two groups, the *S.mansoni*-positive (re-infected and *S. mansoni*-negative (uninfected) group based on Kato Katz assays of 3 stool samples, 2 slides each per person. Both anti-SEA IgE and IgG4 antibody levels were significantlydifferent across the groups (P<0.0001) (Fig. 5.1). Subsequent post-hoc analyses showed that anti-SEA IgE levels were higher in the combined post-treatment group (median, 110.6; IQR, 471.1 AU; P<0.05) and in the schistosomiasis positive post-treatment group (median, 154.9; IQR 577.0 AU; P<0.01) compared to the pre-treatment group (median, 48.5; IQR, 173.2 AU). The converse was true for anti-SEA IgG4 responses which were reduced in the combined post-treatment group (median, 36.0; IQR, 278.6 AU; P<0.0001) and in the schistosomiasis negative post-treatment group (median, 3.7; IQR, 37.1 AU; P<0.0001) compared to the pre-treatment group (median, 134.9; IQR, 685.0 AU).Additionally, the post-treatment schistosomiasis positive group had higher anti-SEA IgG4 levels (median, 117.4; IQR, 553.2 AU; P<0.0001) compared to the schistosomiasis negative group (median, 3.7; IQR, 37.2 AU). Both anti-SWAP total IgG and IgG4 were significantly different across the groups (P<0.001 and P<0.0001 respectively; Fig. 5.2). Post-hoc analyses

revealed that IgG levels were lower in the schistosomiasis negative post-treatment group (median, 53.6; IQR, 80.6 AU) relative to the pre-treatment group (median, 88.9; IQR, 177.6 AU; P<0.05) and the schistosomiasis negative post-treatment group (median, 98.1; IQR, 97.9 AU; P<0.001). Similarly, anti-SWAP IgG4 levels were reduced in the combined post-treatment group (median, 0.0; IQR, 21.3 AU; P<0.0001) and the schistosomiasis negative post-treatment group (median, 0.0; IQR, 0.0 AU; P<0.0001) compared to the pre-treatment group (median, 26.4; IQR, 135.9 AU). Levels of the IgG4 were also higher in the schistosomiasis positive group (median, 5.1; IQR, 51.1 AU) compared to the schistosomiasis negative post-treatment group (median, 0.0; IQR, 21.3 AU; P<0.001; Fig. 5.2).





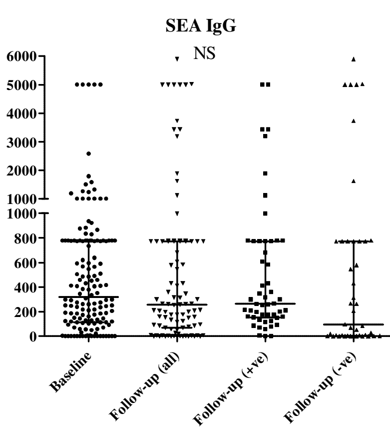


Figure 5.1: Plasma anti-soluble egg antigen (SEA) immunoglobulin (Ig)E, IgG and IgG4 antibody levels. Scatter plots showing antibody levels (arbitrary units) in school children at baseline, 1 year post-treatment (all children), schistosome-egg positive and egg negative groups. Data are presented as dot plots with line at median and whiskers at 75th and 25th percentile and scales for all the graphs are not uniform. Data analysis by Kruskall Wallis and post-test by Dunn's multiple comparison test. *P<0.05, *P<0.001 and ***P<0.0001.

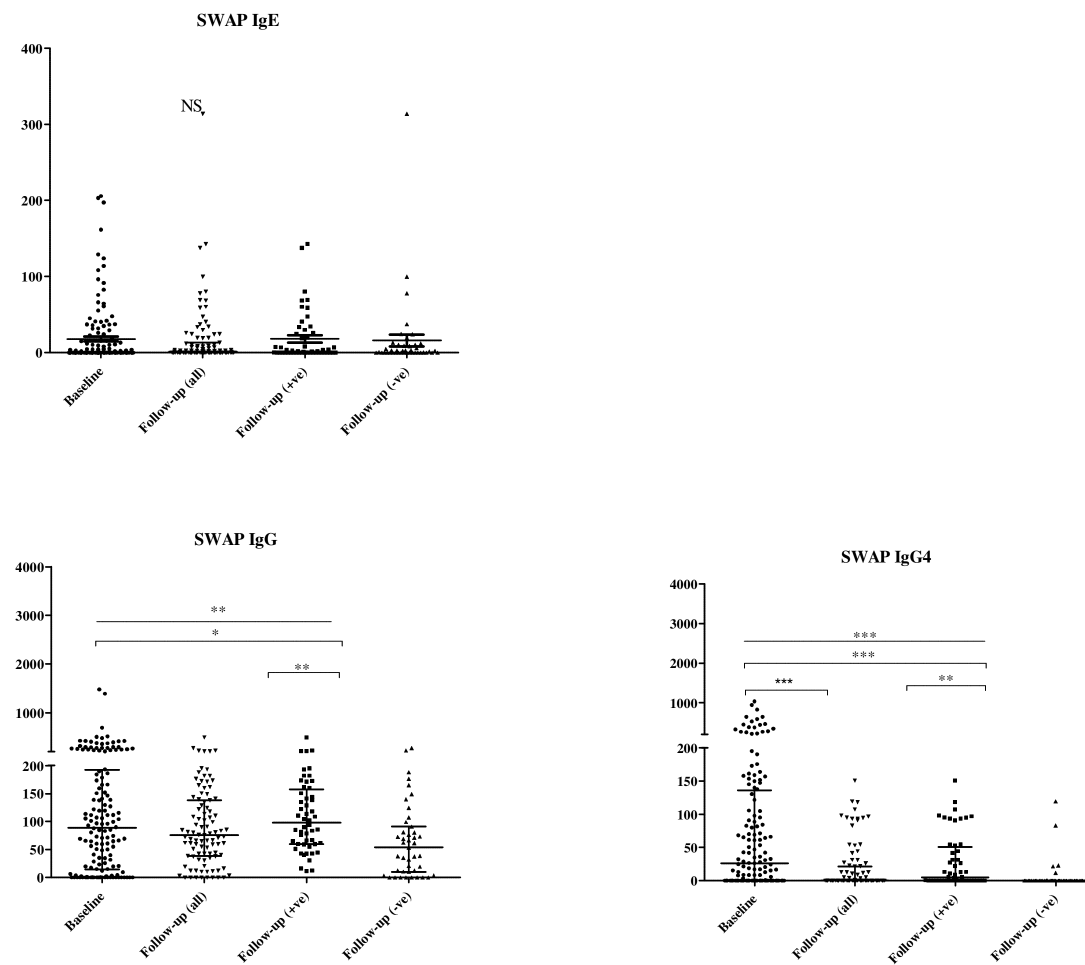


Figure 5.2: Plasma anti-soluble worm antigen prepration (SWAP) immunoglobulin (Ig)E, IgG and IgG4 antibody levels. Scatter plots showing antibody levels (arbitrary units) in school children at baseline, 1 year post-treatment (all children), schistosome-egg positive and egg negative groups. Data are presented as dot plots with line at median and whiskers at 75th and 25th percentile and scales for all the graphs are not uniform. Data analysis by Kruskall-Wallis and post-test by Dunn's multiple comparison test. *P<0.05, *P<0.001 and ***P<0.0001.

5.6.3 Anti-SmTAL antibody responses

Responses to the SmTAL recombinant antigens were generally very low. Anti-SmTAL1 IgE and IgG4 levels were mostly 0, with IgE medians being significantly different across the groups (P<0.05; Fig. 5.3 and 5.4). This difference however turned out to be insignificant following post-hoc analyses. For SmTAL2 responses, only

IgG4 median levels were significantly different across the groups ($P<0.001$) while IgE median levels remained largely unchanged ($P>0.05$). Further analyses showed a reduction in anti-TAL-2 IgG4 medians in the schistosomiasis negative post-treatment group (median, 0.0; IQR, 1.6 AU) relative to the pre-treatment group (median, 16.0; IQR, 183.6 AU; $P<0.001$) and the schistosomiasis positive post-treatment group (median, 1.0; IQR, 319.6 AU; $P<0.05$). Anti-SmTAL-5 IgE medians also differed significantly across the groups ($P<0.0001$). Post-hoc analyses revealed reduced anti-SmTAL-5 IgE median antibody levels in the combined post-treatment group (median, 2.1; IQR, 12.5 AU; $P<0.0001$), in the schistosomiasis positive (median, 1.9; IQR, 10.8 AU; $P<0.0001$) and negative (median, 2.2; IQR, 13.3 AU; $P<0.001$) post-treatment groups compared to the pre-treatment group (median, 10.3; IQR, 22.0 AU) (Fig. 5.3).

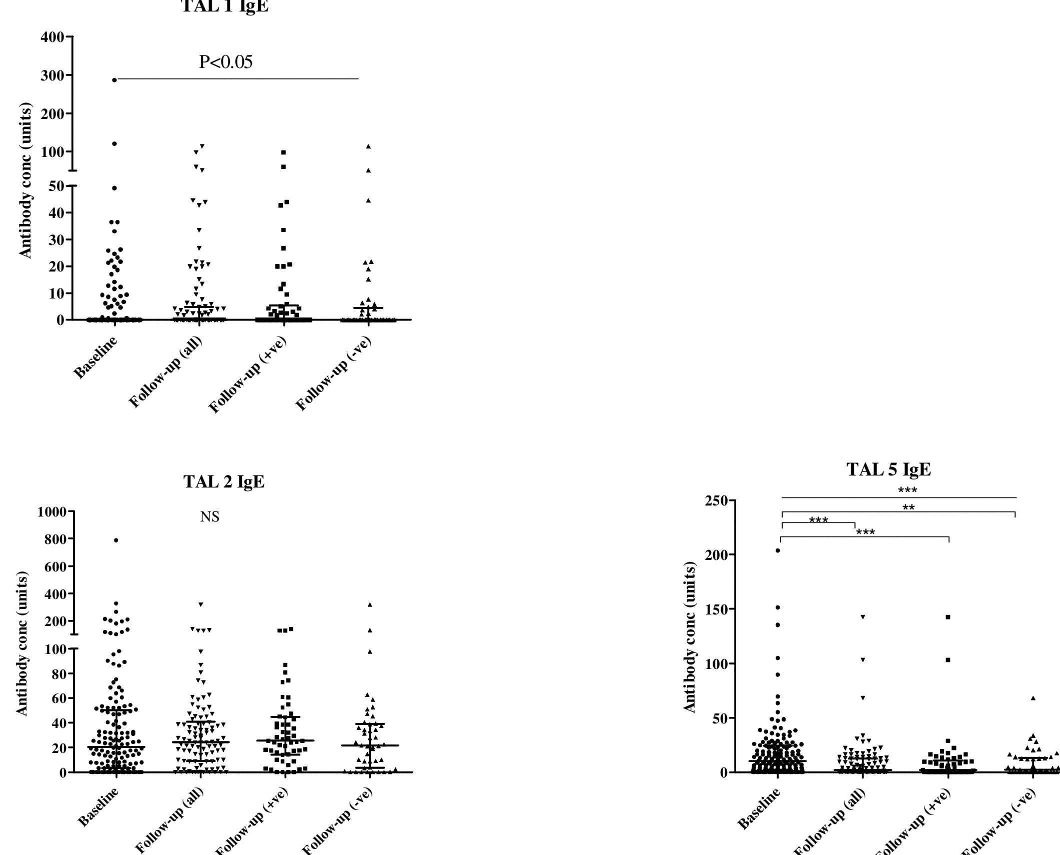


Figure 5.3: Plasma anti-*Schistosoma mansoni* tegument allergen-like (SmTAL) 1, SmTAL 2 and SmTAL 5 immunoglobulin (Ig)E and IgG4 antibody levels. Scatter plots showing antibody levels (units) in school children at baseline, 1 year post-treatment categorised as all participants, schistosomiasis egg-positive and egg-negative. Data are presented as dot plots with line at median and whiskers at 75th and 25th percentile. Each graph has a different scale. Data analysis by Kruskall-Wallis and post-test by Dunn's multiple comparison test. *P<0.05, **P<0.001 and ***P<0.0001.

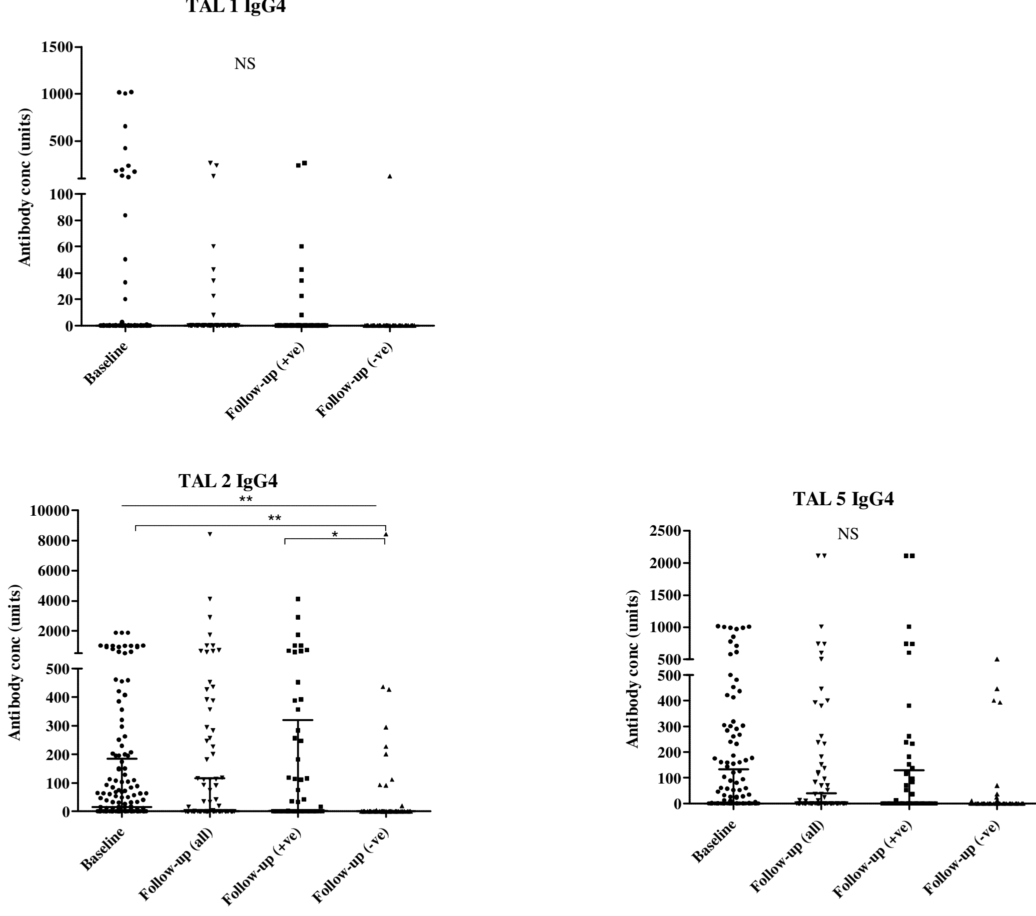


Figure 5.4: Plasma anti-*Schistosoma mansoni* tegument allergen-like (SmTAL) 1, SmTAL 2 and SmTAL 5 immunoglobulin (Ig)G4 antibody levels. Scatter plots showing antibody levels (units) in school children at baseline, 1 year post-treatment categorised as all participants, schistosomiasis egg-positive and egg-negative. Data are presented as dot plots with line at median and whiskers at 75th and 25th percentile. Each graph has a different scale. Data analysis by Kruskall-Wallis and post-test by Dunn's multiple comparison test. *P<0.05, **P<0.001 and ***P<0.0001.

5.6.4 CD23+ B cell proportions

There was an overall significant difference in the proportion of CD23+ B cell levels across the groups (P<0.0001). Further post-hoc analyses showed reduced mean percentage CD23+ B cell levels in the combined post-treatment group (mean 61.7; SEM 1.2; P<0.0001), in the schistosomiasis positive (mean 62.78; SEM 1.6; P<0.05) and schistosomiasis negative post-treatment group (mean 60.2; SEM 1.4; P<0.0001) in comparison to the pre-treatment group (mean 67.4; SEM 0.8) (Fig 5.5).

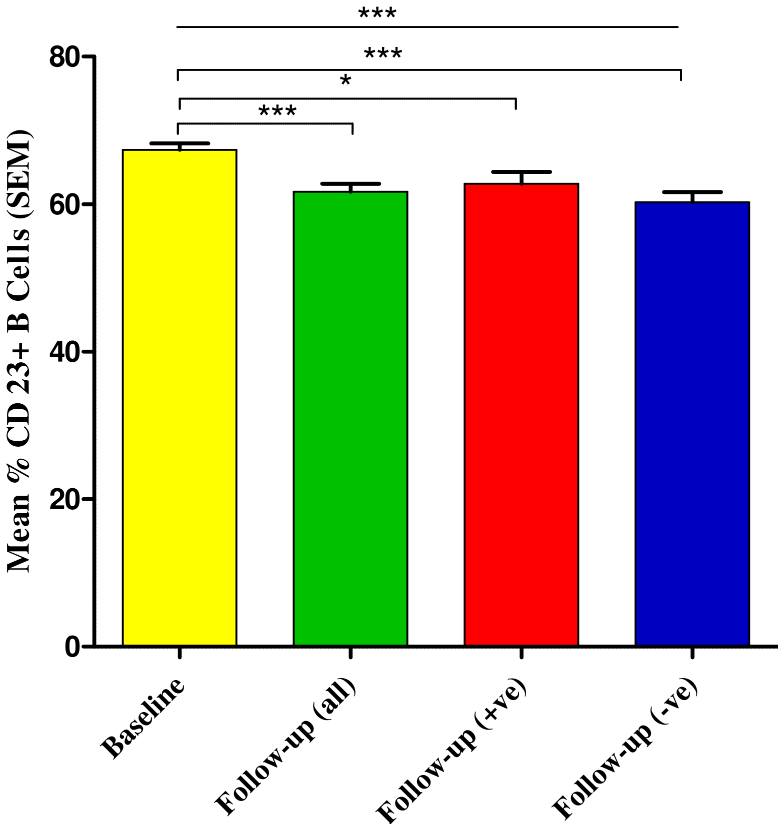


Figure 5.5: Percentage of CD19+ CD23+ B lymphocytess.Bar graph with bars showing mean percentages and standard error of the mean (SEM, error bars) in school children at baseline, 1 year post-treatment categorised as all participants, schistosomiasis egg-positive egg-negative. Statistical analyses by ANOVA, and Turkey's multiple comparison post-test; significance at * P<0.05), **P<0.001 and ***P<0.0001

5.6.5 SEA- and –SWAP specific cytokine levels

Most of the cytokines measured *in vitro*from whole blood cultured with SEA or SWAP did not differ significantly (P>0.05). This was true for IL-10 production in response to SEA and SWAP and IFN-γ produced in response to SEA (Fig. 5.6 and 5.7).However, IL- 13 produced in response to SEA and SWAP and IL-5 produced in response to SEA were significantly different between pre- and post-treatment groups (P<0.01, P<0.0001 and P<0.0001, respectively). Post-hoc analysis for IL-5 showed that the schistosomiasis negative post-treatment group had higher mean levels of this cytokine (mean, 84.9; SEM, 32.1 pg) compared to mean baseline levels (mean, 6.4; SEM, 2.1 pg; P<0.0001) and schistosomiasis positive post-treatment group (mean, 21.7; SEM, 7.3 pg; P<0.001). Soluble egg antigen stimulated mean IL-13 levels were higher in the combined post-treatment group (mean, 137.8; SEM 41.7 pg) compared to mean baseline levels (mean, 19.9; SEM, 4.0 pg; P<0.05). Similarly, mean IL-13 levels produced in response to SWAP were higher in the combined follow-up group (mean, 127.0; SEM, 28.3 pg; P<0.001) and in the schistosomiasis positive post-treatment group (mean, 134.6; SEM, 42.3 pg; P< 0.05) relative to mean baseline levels (mean,29.3; SEM, 6.2 pg).Levels of SEA and SWAP-stimulated IFN-g were marginally increased in the follow-up group compared to baseline levels, though this did not reach statistical significance (P>0.05). (Fig. 5.6 and 5.7).

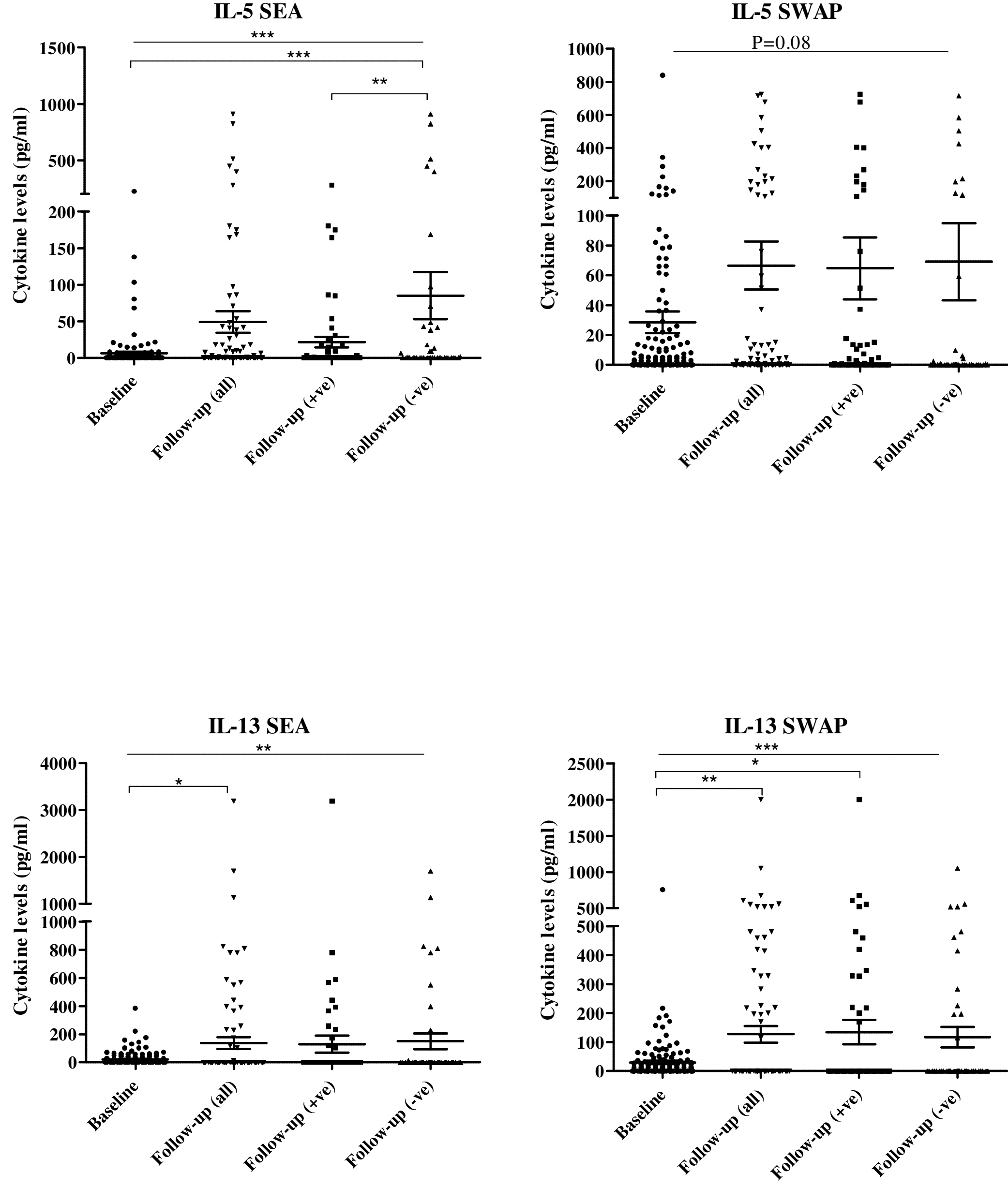


Figure 5.6: Plasma supernatant interleukin (IL)-5 and IL-10 from whole blood cultured with soluble egg antigen (SEA), left panel or soluble worm antigen preparation (SWAP), right panel. Scatter plots showing cytokine levels in school children at baseline and 1 year post-treatment categorised as all participants, schistosomiasis egg-positive and egg-negative. Data are presented as dot plots with line at mean and whiskers at standard error of the mean. Graphs are based on different scales. Data analysis by ANOVA and post-ANOVA test by Turkey's multiple comparison test. *P<0.05, **P<0.001 and ***P<0.0001.

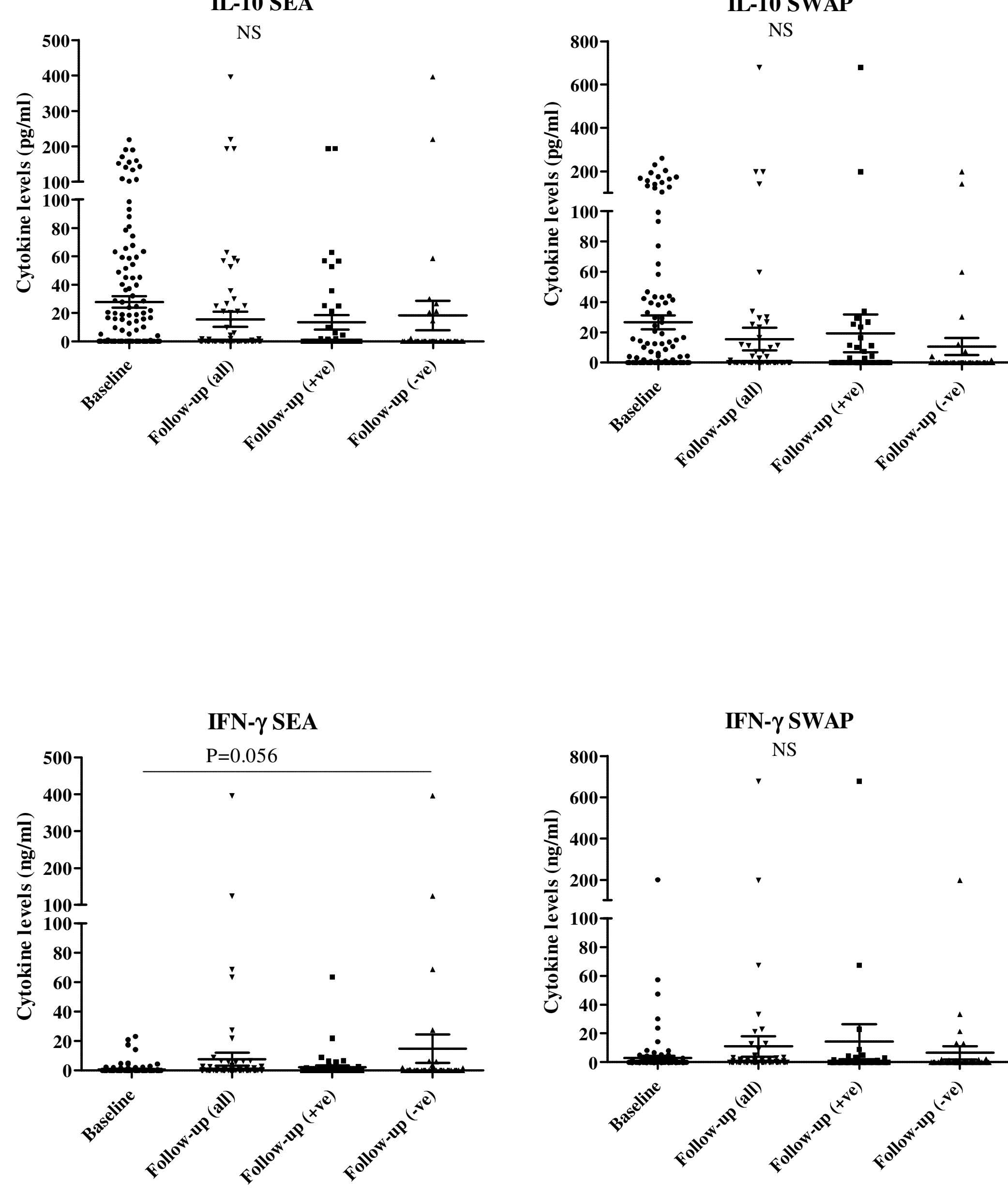


Figure 5.7: Plasma supernatant interleukin (IL)--13 and interferon (IFN)-γ from whole blood cultured with soluble egg antigen (SEA), left panel or soluble worm antigen preparation (SWAP), right panel. Scatter plots showing cytokine levels in school children at baseline and 1 year post-treatment categorised as all participants, schistosomiasis egg-positive and egg-negative. Data are presented as dot plots with line at mean and whiskers at standard error of the mean. Graphs are based on different scales. Data analysis by ANOVA and post-ANOVA test by Turkey's multiple comparison test. *P<0.05, **P<0.001 and ***P<0.0001.

5.7 Discussion

Praziquantel is the drug of choice for the treatment of *Schistosoma* infection in humans and is now widely distributed during mass treatment programs in schistosomiasis endemic areas. In this study, mass treatment of school children with praziquantel in a schistosomiasis endemic area was carried and schistosome-specific immune response determined before and 1 year after treatment in children of the same age. Among the notable findings from the serological responses examined in this study was an increase in anti-SEA IgE in the combined post-treatment group. This is in agreement with findings by Mutapi and group who reported a post-treatment increase in anti-egg antigen IgE from a study involving children infected with *S. haematobium*(Mutapi *et al. 1998).* In this study however, follow-up measurements were done at 18 months and 36 months after treatment. Gomez et al also reported post-treatment increase in anti-SEA IgE, 6 months post-treatment with oxamniquine in a study done in Brazil (Gomes *et al*. 2002). In the current study, anti-SEA IgE levels increased after treatment, and were particularly higher in the schistosomiasis egg positive post-treatment group rather than the schistosomiasis egg negative group. This could be as result of a boost in anti-egg responses following re-infection since egg antigens, unlike adult antigens are continuously exposed to the immune system during chronic infection (Fitzsimmons *etal*. 2006).

Post-treatment increase in anti-worm IgE has been associated with resistance to re-infection. Moira *et al.* reported a 12 months post-treatment increase in anti-SWAP IgE levels which was associated with resistance to re-infection regardless of age (Moira *et al*. 2013). No such median increase in anti-SWAP IgE responses was noted in the current study, and levels of this antibody did not differ between schistosomiasis egg

positive and egg negative group. It has been demonstrated that increase in the protective anti-SWAP IgE and other resistance associated immune responses occur following repeated rounds (sometimes taking 7-9 treatments over several years) of praziquantel treatment, possibly as a result of increased exposure to the immune system of hitherto hidden worm antigens (Karanja *et al*. 2002; Black*et al*. 2010a; Caldas 2000). There has not been any report of praziquantel treatment among children in this age group in Asembo since 2008 when the last study was conducted by our research group (Black*et al*. 2010a) which involved multiple treatments in a 2 year period. It is therefore most probable that this is the first treatment with praziquantel these children were receiving since their initial infection.

There was an overall decline in anti-SEA and –SWAP IgG4 and anti-SWAP IgG responses 1 year post-treatment. It is also noteworthy that these responses were in particular lower in the schistosome egg negative group compared to the egg positive group. Higher levels of SWAP-specific IgG4 have been associated with susceptibility to re-infection and correlated with parasite load in schistosomiasis.This antibody is hypothesised to compete with protective parasite-specific IgE for antigen binding, and therefore its production ameliorates the protective role of IgE (Jiz *et al*. 2009).Others have suggested that protective immunity is more than about absolute levels of worm-specific IgE or IgG4, but rather about the favourable balance between IgE andIgG4(Demeure *et al*. 1993; Hagan *et al*. 1991), and that elevated IgE/IgG4 ratio is more reflective of protective immunity (Figueiredo *et al*. 2012).

Antibody levels to SmTAL proteins in children in this study were largely quite low. This was especially the case for anti-SmTAL-1 IgE and IgG4, which is chiefly expressed in the adult stage of the parasite, with the former being associated with resistance to re-infection (Webster *et al*. 1997; Pinot de Moira *et al*. 2013).The low

anti-SmTAL-1 antibody levels observed at baseline in this study could be due in part to sequestration of the antigens in adult worms. These antigens only get exposed to the immune system after the natural death of the worms or following chemotherapeutic-induced death (Fitzsimmons *et al*. 2012a). It is therefore expected that anti-SmTAL-1 IgE levels would increase after treatment, more so after multiple rounds of treatment after re-infection. One round of treatment however did not result in detectable increases in this response in this study. This is consistent with findings by Moira and others who reported low anti-SmTAL1 IgE in preschool children (Moira *et al*. 2013). Also consistent with this group's findings from this study was higher levels of anti-SmTAL-2 IgE which did not however differ after treatment. This antigen is expressed in all the stages of the parasite, including the egg, and IgE to this antigen tends to be low among chronically infected individuals but higher in recently exposed individuals or younger children (Pinot de Moira *et al*. 2013). Anti-SmTAL-2 IgG4 levels however decreased in the post-treatment group relative to the baseline levels, just as was observed with levels of the antibody against SEA. Similarly, anti-SmTAL-5 IgE levels declined after treatment. This antigen is expressed only in cercariae and in adult worms, and may be similarly sequestered from the immune system just like SmTAL-1. While anti-SmTAL-5 IgE levels were detected in more children than anti-SmTAL-1 IgE, at baseline, median levels were low, above 0.

This study also examined a sub-set of B lymphocytes expressing CD23, the low affinity IgE receptor (FcεRII) that is also expressed on other cell types (Acharya *et al*. 2010). What was observed in this study was that the proportion CD23+ B cells declined slightly after treatment, more markedly in the schistosomiasis egg negative group. This is in contrast to what was observed by our group in a previous study that found an increase in CD23+ B cells in children that were not reinfected as often after

treatment (Black*et al*. 2010a). In this same study however, the increase was examined two years after multiple treatments in 8-10 year old children.Increase in expression of CD23 on B cells has also been linked to resistance to re-infection that develops after multiple rounds of treatment with praziquantel in adults (Mwinzi *et al*. 2009). It may take a longer duration than the 1 year, 1 treatment for up-regulation of the expression of this marker following praziquantel treatment, as Labuda and group similarly did not record any increase in the marker after 6 months of multiple treatments of *S. haematobium* in children (Labuda *et al*. 2013).

Both T_H1 and T_H2 *in vitro* cytokine levels were determined in the children enrolled in this study, at baseline and after treatment. Protective immunity in schistosomiasis infections is associated mainly with elevated SWA-specific IL-5(Roberts *et al*. 1993; Ribeiro *et al*. 2000). In this study however, SWAP-specific IgE increased marginally in the post-treatment group regardless of schistosomiasis status, in contrast to findings in a study involving children and adults in Uganda, in which SWAP IgE levels increased after treatment (Walter *et al*. 2006). It may require multiple rounds of treatment and re-infection to induce elevated IL-5 levels and other immune correlates of resistance to re-infection. On the other hand, SEA-specific IL-5 levels increased after treatment, and were higher in the schistosomiasis egg negative group compared to the egg positive group. This is in agreement to findings in another study involving school children done in Kenya, which reported a post-treatment increase in IL-4, IL-5, IL-9 and IL-13 from SEA-stimulated cultures (Wilson *et al*. 2014).

The regulatory cytokine, IL-10 did not differ significantly after treatment, although there was a marginal reduction post-treatment from both SEA and SWAP-stimulated cultures. This mirrors what was reported by Wilson *et al*(Wilson *et al*. 2014).Interleukin-10 is a key immunomodulatory cytokine during schistosomiasis

infection and more of it is produced by infected compared to un-infected children (van den Biggelaar *et al*. 2000; Meurs *et al*. 2011). In this study, baseline IL-10 levels were marginally higher than post-treatment levels, and for SWAP-stimulated cultures, the schistosome egg positive group had slightly higher IL-10 levels than the egg negative group. This is an indication of parasite-driven IL-10 secretion which is in part responsible for anti-inflammatory responses characteristic of schistosomal infections (van den Biggelaar *et al*. 2000). Another T_H2 cytokine assayed in this study was IL-13, and its levels increased post-treatment for both SEA and SWAP-stimulated cultures, just as was reported by Wilson and colleagues in a previous study involving children (Wilson *et al*. 2014). A similar observation was made with the only T_H1 response examined in this study, that is IFN-γ responses from SEA stimulated cultures increased post-treatment, and noticeably though not significantly in the schistosome egg-negative group. This too is concurrent with what was reported by Wilson and group (Wilson *etal*. 2014), which is indicative of the boosting of immune responsiveness to parasite antigens that occurs at the death of the worm and subsequent exposure of previously sequestered antigens.

In conclusion, this study has found that one round of treatment of schistosomiasis with praziquantel in children did not result in a detectable boost in immune responses associated with resistance to re-infection. While it has previously been reported by our group that frequent treatment with praziquantel over 2 years increases immune responses towards protective levels in children (Black*et al*. 2010a), the same may not be observed after only one annual round of treatment.The reason that this study involved a single annual treatment is because it was meant to reflect what National NTD programs do and therefore examine the impact that is currently being made on millions of African children and will soon be affecting millions more. There is

however, even after only one annual treatment, an early trend towards increased protective responses from this study especially SEA and SWAP-specific IL-5 and IgE against crude and recombinant parasite antigens, thus necessitating a longer study period to monitor the long term effect of annual mass treatment with praziquantel on these responses. Notable also from this study was the apparent decline in parasite-specific IgG4, high levels of which havepreviously been associated with susceptibility to re-infection. The decline was particularly notable among the schistosome egg negative post-treatment group, which may be indicative of the role of IgG4 in neutralizing the protective effect of IgE.

CHAPTER SIX: GENERAL DISCUSSION CONCLUSIONS AND RECOMMENDATIONS

6.1 Discussion

This study examined anti-schistosome immune responses in a cross-section of children before and 1 year after treatment with praziquantel. Prevalence and intensity of schistosomiasisinfection are usually higher among children living in endemic areas compared to adults, and they begin to plateau off and eventually decline in early adulthood. This is an indication of the natural development of resistance to re-infection, which while not resulting in sterile immunity, is nonetheless useful in limiting re-infections. It is hypothesised that the death of worms exposes antigens concealed from the immune system and that this acts to boost protective concomitant immunity against invading schistosomulae. This death of worms is either natural, after worms living for an average 3-10 years, sometimes up to 30 years or as result of treatment (van Dam *et al*. 1996).

In this study, protective responses were profiled in 7-18 year old school children to establish the evidence of protective immunity development and the age group if any when these response begin to appear. Results from this study were equivocal, although the main indications were of a trend towards more responsiveness between 9-11 year old and 12-14 year old age groups for anti-SEA IgE and anti-TAL 1 IgE. The peak percentage of CD23+ B lymphocytes was also in the 12-14 year old age group. The rest of the anti-TAL and –SWAP antibody responses were generally quite low at baseline. In the follow-up study, focus was directed at a more limited age range of 7-13 year olds, encompassing the ages in which some of the anti-IgE responses and CD23+ B cells tended to peak at baseline, with an inclusion of younger children. It was envisaged that any boost in protective anti-schistosome immune responses after

treatment would be better demonstrated in this group. The intent of this study was to mimic the timing and frequency of National NTD Control Programs' MDA against schistosomiasis, in order to see the impact this MDA regimen might have upon school-age children. This is because these programs are now being instituted throughout Africa and while it is known that this will decrease morbidity, we do not yet know its impact on the immune responses of these children.

The baseline serological and cellular responses did not follow the typical age-dependent pattern observed by other studies (Fulford *et al*. 1998; Dunne *et al*. 1992). In these studies however, a wider range of ages are usually examined or primarily focus on adults of different ages. It has also been suggested that development of protective responses is not exclusively dependent on age, but rather on exposure (Black*et al*. 2010b). The range of 7-18 year old children examined in this study at baseline may represent very diverse exposure and infection patterns quite dissociated from their ages.

Based on baseline results and study design, post-treatment responses were examined in 7-13 year old children, with the main recruitment criteria being their participation in MDA at baseline. The main finding of this study is the post-treatment increase in parasite-specific IL-5 and IL-13, while IL-10 and IFN-γ remain unchanged. This is quite unlike what was reported by another Kenyan study involving school children, where all these cytokines from SEA and SWAP-stimulated cultures increased with the exception of IL-10 (Wilson *et al.* 2014). This particular study however was carried out in an area with limited transmission of schistosomiasis while the study was conducted among children in schools very close to Lake Victoria, an area of very high transmission of *S. mansoni*. Other protective responses remained largely unchanged after treatment. Lending credence to suggestions that parasite-specific IgG4 blocks

the protective effects of IgE (Hagan *etal*. 1991) was the observation in this study of lower IgG4 levels in schistosomiasis-egg negative individuals. This therefore may have allowed the protective effect of existing levels of anti-parasite IgE to be effectiveagainst re-infection.

Augmentation of protective responses following treatment could not be demonstrated in this study in the age groups examined. While treatment may have killed worms and exposed previously sequestered antigens thus producing the ‘immunizing effect’ as hypothesised by others (Mutapi *et al*. 1998), a single treatment episode may be insufficient to boost the protective responses. Some of these responses, especially antibody levels may have waned giving the 1 year period of follow-up after treatment. It may therefore take several treatment episodes following re-infections as demonstrated in adults in previous studies(Karanja *et al*. 1997; Black *et al*. 2010a).

The main limitation of this study lies in the design adopted where children not infected with *S. mansoni*(negative controls) were not included to enable establishment of such background responses. This however does not take away the utility of establishing an immunological profile across infected children of different ages that were examined in the study. The repeated cross-sectional design of this study would also not allow us the benefit of following up same individuals over time in order to monitor an individual’s changes in immune responses over time. This study design nonetheless still gave an opportunity to attempt to validate the observation of age-dependent development of immunity seen in individuals living in schistosomiasis endemic areas.

6.2 Conclusions

1. There were no consistent age-specific differences in anti-schistosome immune responses in the 6-17 year old school children infected with *S. mansoni*that were enrolled in this study. Each immune response had its own pattern independent of the age of the children.

2. The immune correlates of protection examined in this study were anti-schistosome IgE, IL-5 and proportion of CD23+ B lymphocytes and their levels before MDA were not associated with age. There was however a general tendency of these responses to begin to increase in the 9-11 year old age group.

3. One round of annual MDA with PZQ did not result in a consistent change in immune correlates of protection in the group of school children examined 1 year post-MDA.

4. Elevated levels of anti-schistosome IgG4 which correlate with susceptibility to re-infection with schistosomiasis was lowest in the schistosomiasis egg-negative post-treatment group, highlighting its probable role as blocking antibody against the protective anti-schistosome IgE.

6.3 Recommendations

a) It would be instructive to follow-up school children and pre-school children in schistosomiasis-endemic areas before MDA roll-out with sufficient numbers in each age to determine pre-treatment anti-schistosome immune responses and their association with age. This could not be clearly delineated in this study due to the limitation of the study design which focused on school children so as to replicate the on-goingnational school based MDA.

b) More studies need to be done on the immune correlates of protection against re-infection of schistosomiasis in different epidemiological settings. This would help to clearly answer the question whether immunity development is age associated. There is need to establish other immunological markers of immunity development to schistosomiasis.

c) The observation that one round of annual MDA does not alter anti-schistosome immune responses makes a case for a sustained annual MDA program targeting all children in schistosomiasis endemic areas. The ministries of health in counties where the disease is endemic should set aside funds for implementing MDA programs both in schools as well as in the community so as also to reach children not in school but who could be carrying infections.

d) The role of anti-schistosome IgG4 as a blocking antibody against protective responses should be investigated further. This is following findings from this study that showed higher levels of anti-schistosome IgG4 in individuals who were schistosome egg positive 1 year post-treatment compared to the egg negative individuals.

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Appendix I: Parent or Guardian Consent Form

STUDY TITLE: Determinants of resistance in human schistosomiasis: School-based treatment, immune responses and outcome

INSTITUTIONS: Kenya Medical Research Institute, Centre for Global Health Research. (KEMRI-CGHR), University of Georgia (UGA) and Centers for Disease Control and Prevention, Division of Parasitic Diseases and Malaria (CDC/DPDM)

PRINCIPAL INVESTIGATORS: Diana M.S. Karanja (KEMRI-CGHR), Dan Colley (UGA), Evan Secor (CDC/DPDM)

CO-INVESTIGATORS:

KEMRI-CVBCR CDC/DPDM UGA

Pauline N.M. Mwinzi Susan P. Montgomery Sarah Nicholson

Bernard Abudho Molly Hyde Jennifer Carter

Liz Ochola Aaron Samuels

Explanation of the purposes of the research

Your child is being asked to take part in a medical research study being performed by the Kenya Medical Research Institute (KEMRI), the University of Georgia, Athens, Georgia, and the Centers for Disease Control and Prevention (CDC), Atlanta, Georgia, USA. It is very important that you understand the following general principles that apply to all participants in our studies:

1) You and your child's participation is entirely voluntary;
2) You may withdraw from participation in this study or any part of this study at any time with no penalty, harm, or loss of access to treatment;
3) After you read about the study please ask any questions that will allow you to understand the study more clearly.

What is Bilharzia

Bilharzia, also known as schistosomiasis is a disease caused by worm parasites transmitted by snails. The snails live in different types of water including ponds, rivers and lakes. People whose activities cause them to come into contact with water where infected snails live and where there may be transmission going on are likely to suffer from the disease. Bilharzia worm parasites enter your body through the skin when you are in the water of a lake, river, stream, or pond. Bilharzia can sometimes be serious or even cause death if not diagnosed and treated properly. In our earlier study in the Asembo Bay area, we found that children in schools closer to Lake Victoria were more likely to have bilharzia than children in schools farther away from the Lake. The most common way to find out if someone has bilharzia is to check for the eggs of the parasite in the stool and urine.

Why do we want to conduct this study:

Although there is medicine available for the treatment of bilharzia, many people still are not able to get it, because they either do not know about it, or they live too far from the hospitals. The World Health Organization recommends that children who live in areas where there is a high prevalence of bilharzia should get the medicines. Scientists still do not know how often children should get the medicines to protect them from the effects of the disease. We are therefore interested to find out how often children who are at risk of getting bilharzia should get treated to keep them from being sick and if treating them a certain number of times will keep them from getting the disease again in the future. This will help scientists be able to develop methods of controlling the disease.

What is important for you to know.

To do this study, we will need to study some of your child's urine, feces and blood. We will first collect stool, urine, and a small amount of blood from a fingerstick. Some of the blood and urine will be tested at the school site. The stool and the rest of the blood and urine will be returned to the laboratory for preparation and other studies. While we are testing for bilharzia, we will also test for anaemia, intestinal worms (roundworms, whipworms and hookworms), and malaria. Your child will be assigned a study number, and the links between the name and number, and all data collected through use of stools, urine, and blood, will be kept confidential. None of the information that we collect will be told to other people in your village. We will just use the information to find out about this disease, and the best way to protect people from suffering the effects of the disease.

If we find that your child has bilharzia, we will do additional tests on your child. These tests will include: 1) asking your child for 2 more stool samples; 2) using a machine that uses sound waves to find out if the bilharzias has caused any damage to your child's spleen or liver; 3) measuring your child's height, weight, and mid upper arm circumference; 4) measuring your child's physical fitness using a shuttle run test; 5) asking you and your child some questions about how healthy they feel and how healthy you think they are; and 6) collecting 8ml (less than one tablespoon) of blood from your child's arm. If we find that your child has anaemia or malaria, they will be offered treatment whether or not they take part in this aspect of the study. All children will be offered treatment for bilharzia and intestinal worms.

We would like to investigate how the treatment of bilharzia can be conducted so as to reach all communities and community members who are at risk of getting the disease. Your child may not get any direct benefits from being in this study but your child's participation will help us determine the best approach for controlling bilharzia in your community. Although your child will receive treatment for bilharzia and other worms, this treatment is also available at the government hospital.

This study is expected to last about 5 years. You can decide if you want your child to take part in this study. Taking part in this study will not cost you or your family anything. Your child may also leave the study at any time. You can leave for any reason without any problems.

Who Can Participate In The Study:

We can include your child in the study only if you give permission for him/her to participate, and if your child agrees to participate. We shall include children over the age of 5 and up to age18.

Risk involved

The risks or hazards to your child if she/he takes part in this study are minimal. There is the minor discomfort while drawing blood. To minimize any risk, hazard or

discomfort during our study, the blood will be obtained from your child's finger or arm in a sterile way by well trained staff.

Questions about research

If you have any questions about this study, you may contact Dr. Diana Karanja at the Kenya Medical Research Institute, Kisumu Tel; 057-2022929 during the study and in the future. If you have concerns about human rights, ethics and welfare issues you may contact Dr. Rashid of the National Ethical Review Board, Kenya Medical Research Instititute; Tel; 020-722541.

PARENTAL/GURDIAN PERMISSION

I, Mr./Mrs./Miss ____, being a person aged 18 years and over and being the lawful/legal guardian of: Mr/Miss (Child's name) ______________ voluntarily agree that my child may be included in the study which I have read or has been read to me. I have been made to understand the implications and benefits of the tests and treatments I accept the tests and treatments to be carried out. I understand that I may withdraw him/her from the research at any time, for any reason, without any penalty or harm. All the above conditions have been explained to me in the ___________ language in which I am fluent.

_____________________________________Age of child________

School name ___________________________Village___________________________

_______________________________________ Parent's/Guardian's signature

_______________________________________ Date

_______________________________________ Place

_______________________________________ Person Obtaining Consent

_______________________________________ Witness

Specimen Storage/Export

Some blood, urine or stool samples obtained from your child may be useful for further development of tests to detect schistosomiasis or see how it makes people sick. For

this, we will need to store the blood or serum for a longer time and possibly send it to the United States. Your child's name will not accompany the specimen if it is stored or exported from Kenya. Is it okay to store and/or export your child's blood or stool samples? ________Yes ________No

Parent's/Guardian's signature ____

OFFICIAL STAMP

Consent form in Dholuo

OBOKE MAR AYIE
WI NONRO: Rapim Ogira mar del mar geng'o aremo mar dhano: Thieth mitimo e sikunde, ogira mar del kod gigo ma thieth kelo

MIGEPE: Migao mar nonro mar tuoche dhano mar Kenya, bat nonro mar ngima dhano piny ngima (KEMRI-CGHR), Mbalariany mar Georgia (UGA) kod migao mochung' ne nono kendo geng'o touche mar piny Amerka, bade mar tuoche mag kute kod maleria (CDC/DPDM)

JOTEND NONRO: Diana M.S. Karanja (KEMRI-CGHR), Dan Colley (UGA), Evan Secor (CDC/DPDM)

JO NORO MAMOKO

(KEMRI-CVBCR)	(CDC-CPD)	UGA
Pauline N.M. Mwinzi	Susan P. Montgomery	Sarah Nicholson
Bernard Abudho		Jennifer Carter
Liz Ochola		

Ler Ewi Gima Omiyo Itimo Nonro

Ikwayo nyathini mondo odonji e nonro mar tueche dhano ma itimo kod Migao mar nonro mar tuoche dhano mar Kenya (KEMRI), Mbalariany mar Georgia, ma nitie

Athens, Geogia, kod migao mochung' ne nono kendo geng'o touche mar piny Amerka, bade mar tuoche mag kute (CDC) mawuok Atlanta, Georgia, piny Amerka. Ber ahinya mondo iwinj makare weche mapiny kaegi, ma chalre ne jogo duto mabedo e nonrowa:

1) Donjo mari kod nyathini e nonro en kuom yie mari iwuon;
2) Inyalo weyo bedo e nonro kata e bad nonroni moro amora saa asaya ma onge hinyruok, wang' kata koso yudo thieth;
3) Ka isesomo twak ewi nonro to penj penjo moro amora mabiro miyo iwinj nonroni ma kare.

Bilhazia en Ang'o

Bilhazia, ma bende iluongo ni Aremo, en tuo ma ikelo kod kute ma ilando gi kamnio. Kamnio gi dak e pige mopogore opogore kaka yao, aora, kod dho nam. Jogo ma tijegi chuno donjo e pige man gi kamnio ma oting'o kutegi nyalo yudo kutegi, kendo yot mondo gibed gi bilhazia. Kute makelo bilhazia donjo gi e pien dendi ka in ei pi mar nam, aora, kata yao. Bilhazia seche moko nyalo bedo makoch mi kel kata tho ka ok ofwenye kendo othiedhe malong'o. e nonrowa mokalo mane watimo e hosi mar Asembo Bay, ne wayudo ni nyithindo masomo e sikunde machiegni kod nam Lolwe ne hinyo bedo gi bilhazia moloyo mago masomo mabor kod namb ni. Yo ma ifwenyogo ahinya ka ng'ato nitie kog bilhazia en rango ka tong kudni ni nitie ei oko kod lach.

Ang'o Ma Omiyo Wadwaro Timo Nonroni:

Kata obedo ni nitie yath ma inyalo thiedh godo Bilhazia ji mang'eny pod ok nyal yudo thieth, nikech nyalo bedo ni ok ging'eyo, kata nikech gidak mabor gi kuonde thieth. Rirwuok mar piny ngima mochung' ne ngima dhano (WHO) dwaro ni nyithindo modak kuonde mantie bilhazia mangeny e oganda onego yud thieth. Jolony pod ok ong'eyo ni to nyithindo onge thiedhi bang' kinde maromo nade mondo okonygi geng'o rach mawuok e tuoni. Ma ema omiyo wadwa temo fwenyo thuolo ma nyithindo ma yot mondo oyud bilhazia onego thiedhie mondo omon gi bedo ma tuo, kendo ka thiedhogi kuom ndalo moko mobidhi nyalo konyogi geng'o tuoni e kinde mabiro. Mae biro konyo jolony keto okenge mag gayo tuoni.

Gigo maber mondo ing'e

Mondo watim nonroni, wabiro pimo lach, oko kod remb nyathini. Wabiro kuongo kawo oko, lach kod remo matin ahinya ewi lith lwedo. Moko kuom remo ibiro pim e sikul. Oko kod remo modong' ibiro ter e kar nonro (lab) mondo olosgi kendo otimnegi nonro mamoko. E seche ma warango bilhazia, wabiro bende rango tin mar remo gi kute moko miyudo e ich (njokni) kod maleria. Nyathini ibiro miu namba mag nonro, kendo tudruok ma e kind nambagi kod nying, bende ler duto ma owuok kuom lach, remo kod oko ibiro kan mopondo ahinya. Wabiro mana tiyo gi wechegi maondo wang'e mang'eny ewi tuoni, kod yo maber mogik mar geng'o chandruok ma ikelo kod tuoni.

Ka wayudo ni nyathini nitie gi bilhazia, wabiro timone pim moko kendo. Magi biro bedo: 1) kwayo nyathini oko 2 moko; 2) tiyo kod gi lony ma sani ma nyalo fwenyo

hinyruok e ma bilhazia nyalo kelo e tako kata chuny nyathini; 3) pimo bor, pek, kod lach mar kor bad nyathini; 4) pimo rikni mar nyathini ka itiyo gi ng'wech ma adunda adunda; 5) penjo nyathini kaka uwinjo ngima dende kod kaka oparo ni ngima dende nitie 6) kawo 8 ml (matin ne ojiko mar chiemo) mar remo e bad nyathini. Ka wayudo ni nyathini rembe tin kata ni en kod maleria to wabiro thiedhe, bed ni entie kata oonge e badni mar nonroni. Nyithindo duto biro yudo thieth mar bilhazia kod njokni mag ich.

Wadwaro manyo kaka inyalo tim thieth mar bilhazia mar mondo omi ochopi ne ogendni duto kod ji duto ma nitie e ogendni duto man kuonde ma tuoni landore ahinya. In kod joodi nyalo bedo ni ok bi bedo gi yuto ma achiel ka achiel kaa e nonro to bedo ne nonro biro konyowa nwang'o yo maber mogik mar gayo bilhazia e ogandau. Kata obedo ni nyathini biro yudo thieth mag bilhazia kod njokni mag ich, thiedhgi bende yudore e kuonde thieth mag piny owacho.

Nonroni duto igeno ni biro kawo gimoro higni 5. Inyalo yiero ka idwaro ni nyathini obed e nonroni. Bedo e nonroni ok bi miyi kata joodi garama moro amora. Nyathini bende nyalo bende weyo bedo e nonro saa asaya. Onyalo wuok nikech wach moro amora maonge rach ma oyudo e wuok.

<u>Ng'ano Manyalo Bedo E Nonro:</u>
Wanyalo rwako nyathini e nonro mana ka ichiwo thuolo mondo odonji, kendo ka oyie donjo. Wabiro rwako nyithindo ma hikgi ni e kind 5 kod 18.

<u>Hinyruok Ma Dibedi E Nonro:</u>
Hinyruok kod rem ne nyathini ka odonjo e nonroni tin mokalo. Nitie rem moro matin ahinya e thuolo ma ikawoe remo matin e lith lwete kata e bade. Mondo odwok piny rem kata hinyruok e nonrowani, remo ibiro kaw e yo maler gi jogo motiegi malong'o.

<u>Penjo Ewi Nonro</u>
Ka in kod penjo moro amora ewi nonroni, inyalo tudori gi Lkt. Diana Karanja ma nitie KEMRI, Kisumo. Namba mar sime mare en 057-202-2929. Ka in kod penjo ewi adiera mag dhano kaluwore gi bedo e nonroni, yie itudori kod Lkt. Rashid ma nitie e bad KEMRI ma ochung ne puodho ratiro mar nonro (National Ethical Review Board) Nairobi; sime- 020-722541.

THUOLO MOCHIW KOD JANYUOL/JARIT NYATHI

An, Migosi/Mikayi/Nyadendi______________________________________, bedo ni hika ohingo 18 kendo nikech an e jarit machik opuodho mar: Rawera/nyako (nying nyathi) ____Hike________________ Nying sikul_____________________________________ Gweng'_________________________________ achiwo thuolo ne lakteche Diana Karanja, Amma Semenya gi Evan Secor mondo omi girwaki nyathi e nonro midwaro tim, ma oler e chenro ma osepimna mi awinjo. Bende osemiya ng'eyo weche kod ber mag pim kuom rawera/nyako_______________________________________. Ayie ni

pim mondo otim. Ang'eyo ni anyalo golo nyathi oko e nonro saa asaya, ne wach moro amora, maonge kuor kata rach moro amora maluwa. Weche ma malo kaegi duto olerna gi dho_______________________________, ma awinjo maber.

__________________________________ Hik nyathi___________

Nying sikul ________________________

Gweng'_________________________________

__________________________________ Sei mar janyuol/jarit

__________________________________ Tarik

__________________________________ Kanye

__________________________________ Ng'at mokawo ayie

__________________________________ Janeno

<u>Kano kata oro remo loka</u>

Mamoko kuom remo, lach kata oko ma oa kuom nyathini nyalo bedo gi ber mar medo loso yore mag fwenyo bilhazia kata maleria. Nikech ber ni, wabiro kano remo kuom thuolo ma lach kendo wanyalo oro loka Amerka. Nying nyathini ok bi bedo e gik ma okan kata ma ioro ka oa Kenya. Bende iyie mondo okan/oor loka remo kata oko mar nyathini?

____________ Ee ____________ Ooyo

Sei mar Janyuol/Jarit_____________________________________

<u>Ayie mar Nythindo:</u>

Wakwayo ni wapim pek, bor kod lach mar kor badi, kedo wago remo matin e badi, kod lach gi oko matin, kendo wathiedhni bilhazia, maleria to gi njokini mag ich ka in kodgi. Wabiro bende goyo iyi picha gi gino miluongo ni*ultrasound,* kendo keti mondo iringi agonda ka ng'ato matugo, kod miyi penjo moko matin ewi kaka iwinjo. Ok ochuni timo ma ka ok idwar, mak mana ni onge rach moro amora ka iyie. Onyalo konyi. Be iyie mondo watim pimgi e dendi, kendo ni ibiro muonyo yath?

____________ Ee ____________ Ooyo

Nying nyathi ______________________________________

Sei kata alama mar lwet nyathi________________________

Ng'at mokawo ayie ________________________________

Sei ______________________________________

Tarik________________

Janeno ______________________________________

Tarik________________

Sei mar Janeno _____________________________________

SITAMP MAKARE

Appendix II: Laboratory protocols

<u>Whole Blood Cultures</u>

1. Protocol for 30 patients bled/day.
2. Make up 220ml media: 2.2ml L-glut, 2.2ml Pen-strep, 215.6ml RPMI
3. Label 30 15ml conicals, one for each patient.
4. Place 7ml media in each conical.
5. Add antigens to the wells of 24-well culture plate

    3 day plate:

        control (no antigen)

        PHA (3.75ul of 1mg/ml stock for 2.5ug/ml final concentration)

    5 day plate:

        control (no antigen)

SEA (5ul of 1.5mg/ml stock for 5ug/ml final concentration)

SWAP (2.5ul of 6mg/ml stock for 10ug/ml final concentration)

6. Add 1.75ml blood to 7ml media in each conical, mix well.
7. Place 1.5ml diluted blood into each well.
8. For harvesting sups, pipet off supernatants and place in a properly labeled cryovial.

Staining Whole Blood

Set up experiment with all the proper controls.

1. Unstained control to set autofluorescence.
2. Either isotype controls or FMO controls for each color being used to account for any signal due to nonspecific “stickiness”.
3. Fluorescence controls: single stained tube for each antibody being used. These are used to set compensation.

Stain whole blood.

1. Add Abs (at proper dilution, diluted in FACS wash buffer) to cells.
2. Pipette 100 ul anticoagulated (Heparin) blood into polystyrene culture tube.
3. Vortex at low speed for 3 seconds and incubate at room temperature for 30 minutes. Protect samples from light during this procedure.

4. Add 2 ml of 1X FACS Lysing Solution (10X lysing solution diluted in deionized water) to each tube.
5. Vortex to mix cells for 3 seconds and incubate at room temperature for 10 minutes. Protect samples from light during this procedure.
6. After incubation, centrifuge tubes at 300X g for 5 minutes at room temperature.
7. Aspirate the supernatant, leaving a small amount of fluid (about 50 ul) in the tube. Avoid disturbing the pellet.
8. Wash the cells 2 times in the following manner:
9. Add 2 ml FACS wash buffer (PBS, 2% FBS, 0.09% azide) to each tube. Vortex to suspend cells for 3 seconds, centrifuge at 200X g for 5 minutes at room temperature. Aspirate the supernatant, leaving a small amount of fluid (about 50ul) in the tube. Avoid disturbing the pellet.
10. Add .7 ml of 1% paraformaldehyde (in PBS) to each tube. Vortex at low speed for 3 seconds.
11. Store prepared tubes at 2° to 8°C in the dark until flow cytometric analysis is performed. Analyze cells within 24 hours of staining.

Anti-TAL antibody ELISA Protocol

1. **a) Reagents and supplies**
    - i. TAL antigens (TAL1, TAL2 and TAL5), DPDM, CDC
    - ii. Serum controls:
        - a. Negative: Normal human serum
        - b. Positive: Positive control serum
    - iii. Mouse anti-human IgG4-HRP (clone: HP6023; Southern Biotech cat# 9190-05)
    - iv. Mouse anti-human IgE-HRP (clone: B3102E8; Southern Biotech cat# 9160-05)
    - v. 0.1M Sodium bicarbonate buffer pH 9.6
    - vi. 0.1M PBS pH 7.2
    - vii. Tween-20
    - viii. Dry milk powder
    - ix. TMB
    - x. Immunlon 2 HB plates

b) **Standards preparation**

Prepare 9 serial 2-folded dilutions of SWAP positive control serum in normal human serum (#1-#9; Undiluted-1:256). Aliquot standards to multiple vials (100 ul/vial) to

prevent protein degradation due to repeat freeze/thaw procedure. Store the standards in -80^0C.

** Arbitrary units can be assigned to the standards for quantitative studies.

c) **Reagent preparation**

i. Working antigen solution (2 ug/ml for TAL1; 4 ug/ml for TAL2 and Tal5)

Antigen	Ag stock [conc]	Ag stock vol. needed For one 96-well plate
TAL1	0.887 mg/ml	24.80 ul
TAL2	1.603 mg/ml	27.44 ul
TAL5	1.208 mg/ml	36.42 ul

Dilute antigen in 0.1M Sodium bicarbonate buffer (pH 9.6) to working concentration of 2 ug/ml for TAL1 and 4 ug/ml for TAL2 and TAL5. Prepare 11 ml of working antigen solution needed for one 96-well plate. Stir working antigen solution using a stir bar for ≥10 min at room temperature.

**Aliquot antigen stock solution to multiple vials (200ul/vial) to prevent protein degradation due to repeat freeze/thaw procedure.

ii. PBS/tween (0.01M PBS, pH 7.2/0.3% Tween-20)

1 liter PBS + 3.0 ml Tween-20; prepare fresh on the day of assay

iii. PBS/Tw-milk (5% milk in PBS/Tw)

100 ml PBS/Tw + 5g Dry milk powder; stir for 5 minutes before use.

iv. **Working antibody conjugate solution**

Mouse anti-human IgG4-HRP (1:8000, 1.3ul per plate)

Mouse anti-human IgE-HRP (1:1000. 11ul per plate)

Dilute antibody conjugate in PBS/Tw; prepare 11 ml per plate and stir for 10 minutes before use.

v. **Working TMB substrate**

30 minutes prior to the addition to assay wells, aliquot required volume (11 ml/plate) of TMB substrate and keep from light at RT

vi. Stop Solution (1N Sulphiric acid)

Add 5.55 ml concentrated H_2SO_4; store solution at RT.

2. PROCEDURE

a. Sensitization –Coating 96 wells plate with antigen
   i. Place the diluted antigen solution in trough. Add 100 ul into each well using multi-channel pipette.
   ii. Seal the plate with adhesive plastic sealer and incubate for 2 hours or longer at room temperature (RT) on the mini-orbital shaker at speed 5.
   iii. Decant the unbound antigen, wash the wells with 200 ul of PBS/Tw. Decant wash buffer and repeat this step a total of 5 times. Remove excess liquid from wells by blotting the plate upside down on paper towels.

b. Antibody incubation

Standards and controls are assayed as single sample, while test sera are assayed in duplicate. Serum dilution use in IgG4 ELISA assays is 1:50, and serum dilution for IgE ELISA is 1:20.
   i. 100ul PBS/Tw/milk is added to the blank well.
   ii. Add 2 ul or 5 ul of standard, control r test sample in the appropriate well, and then add either 98 ul or 95 ul of PBS/Tw-milk using a multichannel pipette. The finala volume of the serum sample should be 100ul.
   iii. Seal the plates with adhesive plastic sealer and incubate the plate at RT for 30 minutes at orbital shaker at speed of 5.
   iv. Decant the unbound sera, wash the wells with 200 ul of PBS/Tw. Decant was buffer and repeat this step a total of 5 times. Remove excess liquid from wells by blotting the plate upside dwon on paper towels.

c. Conjugate incubation
   i. For each plate, prepare 11 ml of diluted conjugate in PBS/Tw (anti-IgG4 at 1:8000 or anti-IgE at 1:1000). The conjugate dilution is predetermined and used in excess. Prepare the conjugate in a beaker and stir using a stir bar until well mixed, for about 10 min.
   ii. Add 100 ul to each well using a multichannel pipette, place a piece of adhesive plastic sealer on the 96-well plate, and incubate for 30 minutes at RT on the mini-orbital shaker at speed of 5.
   iii. Decant the unbound conjugate, was the wells with 200 ul of PBS/Tw. Decant was buffer and repeat this step a total of 5 times. Remove excess liquid from the wells by blotting the plate upside down on paper towels.

d. Substrate incubation
   i. Using the multichannel pipette, dispense 100 ul/well of prewarmed TMB.
   ii. Incubate for 10 minutes at RT on the mini-orbital shaker at speed 5.
   iii. Add 100 ul/well of stop solution (1N H_2SO_4).

iv. Measure sample OD at 450 nm on the thermomax microplate reader or similar reader using the SOFTmax prosoftware if available.

Anti-SWAP and Anti-SEA ELISA: (July 2014 for ELISAs in Kenya, KEMRI-CGHR, NTD LAB)

Plate Instructions for up to 41 samples: I day Protocol

1. Label 6 Immulon 2HB plates, two plates per isotype, three for either SEA or SWAP (IgE, IgG, and IgG4).
2. Incubate 100 ul/well of 5ug/ml SWAP, 0.625ug/ml SEA (for IgG/G4) and 2.5ug/ml SEA for IgE in 0.1M Carbonate Buffer (pH 9.6) for 2 hours at RT, covered with parafilm and on a Titre Plate shaker at 1.5 speed.
3. Decant the unbound antigen, wash the wells with 200 ul of PBS/Tw. Decant wash buffer and repeat this step a total of 5 times. Remove excess liquid from wells by blotting the plate upside down on paper towels
4. Add 100ul per well of each sample in duplicate and each standard in single well. Refer to sample plate set-up and standard preparation. Seal the plates with adhesive plastic sealer or parafilm and incubate the plate at RT for 30 minutes on Titre Plate shaker at speed of 1.5.
5. Decant off samples and standards and wash each plate with wash buffer 3-5 times as above.

6. Add 100ul per well of anti-isotypes, varying dilutions. Incubate plates at room temperature for 30 minutes, covered with parafilm on the Titre Plate shaker. During this step, take TMB substrate solutions out of 4C and measure 30ml of each of the two components of into 50 ml conical tubes so as to reach room temperature. Also work on Softmax set-up during this time.
7. Decant off anti-isotype solutions from the plates and wash each plate with wash buffer 3-5 times.
8. Just before adding substrate, mix equal parts of Solution A and Solution B, and then add 100ul per well of the mixed substrate to each well
9. Incubate for 10 minutes for IgE plates, and for 5 minutes for IgG and IgG4 at RT on the mini-orbital shaker at speed 5.
10. Add 100 ul/well of stop solution (1N H_2SO_4).
11. Measure sample OD at 450 nm on the thermomax microplate reader or similar reader using the SOFTmax prosoftware if available.

Sample and Positive standards Preparations: (Including Unknowns, Normal Human Sera and ELISA controls)

a. Prepare standards (Tubes 1-8)
    i. Add 900µl 1XPBS + 0.3% tween 20+ 5% milk to Tube 1 and 450µl of the same to each of Tubes 2-8.
    ii. Dilute 18µl positive plasma pool in Tube 1 for IgE and 2.5ul each in tube for IgG/G4; vortex.
    iii. Transfer 450µl from Tube 1 to Tube 2; vortex.
    iv. Continue 1:2 serial dilution for Tubes 3-7.
    v. Tube 8 should 0 positive plasma pool
    vi. Prepare another set-up of 8 tubes for IgE standards, and following similar procedure, make a 2-fold serial dilution beginning with 600ul of 1XPBS + 0.3% tween 20+ 5% milk. Subsequent tubes should have 300ul of the same solution. Add 30ul of positive plasma pool to tube 1, and transfer 300ul to subsequent tubes.

2. After deciding which samples to run, pull the heparin plasma samples from the freezer and allow thawing.
3. Arrange micro-test-tubes in two separate racks according to sample arrangement order on the plate template, one rack for IgE samples and another for IgG/IgG4.
4. Measure and transfer 500ul and 1000ul of 1XPBS+0.3% tween 20+ 5% non-fat milk powder to each of the micro-test-tubes in the IgE and IgG/G4 racks respectively.
5. Vortex and transfer 25ul of each sample to its respective micro-test-tube in the IgE rack and 20ul of each sample to its respective tube in the IgG/G4 rack. This is to prepare dilutions of plasma samples as follows:

    IgE plasma 1/20
    IgG/G4 plasma 1/50

6. Be sure samples are thoroughly mixed (vortexed) and add in duplicate to the plates accordingly. Pipette up and down several times in each micro-test-tube

using a multi-channel pipette before loading samples in their respective wells in the plate.

Anti-isotype preparations:

1. Dilutions are done in 1X PBS+0.3%Tween 20 as follows. Estimate about 10ml to cover one plate.

IgE anti-isotype	1/1000
IgG anti-isotype	1/50000
IgG4 anti-isotype	1/50000

Solution Preparations:

Carbonate Buffer: In 1L beaker mix 45.3ml solution A and 18.2ml solution B (not to be confused with substrate solution A and B, these are different). Check pH. Adjust with solution A or B until pH 9.6 is achieved. Fill to 1L with distilled water.

Solution A: 42g NaHCO3 (sodium bicarbonate) dissolved in 500ml distilled water (1M)

Solution B: 52g Na2CO3 (sodium carbonate, anhydrous) dissolved in 500ml distilled water (1M)

Wash Buffer (0.05%): To 2L 1X PBS, add 1ml Tween 20. Mix thoroughly.

Plasma sample diluent: To 100ml 1X PBS, add 300ul Tween 20 stir and 5g non-fat dry milk powderto mix.

Plate set-up

	1	2	3	4	5	6	7	8	9	10	11	12
A	STD1	STD2	STD	STD	STD	STD	STD	STD	Pos C	PosC	NHS	NHS
B	Un1	Un1	8	8	15	15	22	22	29	29	36	36
C	2	2	9	9	16	16	23	23	30	30	37	37
D	3	3	10	10	17	17	24	24	31	31	38	38
E	4	4	11	11	18	18	25	25	32	32	39	39
F	5	5	12	12	19	19	26	26	33	33	40	40
G	6	6	13	13	20	20	27	27	34	34	41	41

H	7	7	14	14	21	21	28	28	35	35	Blank	Blank

Appendix III: KEMRI ERC Study Approval

KENYA MEDICAL RESEARCH INSTITUTE

P.O. Box 54840-00200, NAIROBI, Kenya
Tel (254) (020) 2722541, 2713349, 0722-205901, 0733-400003; Fax: (254) (020) 2720030
E-mail: director@kemri.org info@kemri.org Website:www.kemri.org

KEMRI/RES/7/3/1 **February 6, 2012**

TO: **DR. DIANA M. S. KARANJA (PRINCIPAL INVESTIGATOR)**

THROUGH: **DR. JOHN VULULE**
THE DIRECTOR, CGHR,
KISUMU

FORWARDED 8/2/12 DIRECTOR CENTRE FOR GLOBAL HEALTH RESEARCH

Dear Madam,

RE: SSC PROTOCOL No. 2125 – 3RD REVISION (*RE-SUBMISSION*): DETERMINANTS OF RESISTANCETO SCHISTOSOMIASIS IN THE HUMAN HOST: SCHOOL-BASED TREATMENT, IMMUNE RESPONSES AND OUTCOME

Reference is made to your letter dated February 2, 2012. We acknowledge receipt of the revised research proposal on February 6, 2012.

This is to inform you that the Committee determines that the issues raised at the initial review and on 28th January 2012 are adequately addressed. Consequently, the study is granted approval for implementation effective this **6th day of February 2012** for a period of one year.

Please note that authorization to conduct this study will automatically expire on **February 4, 2013**. If you plan to continue data collection or analysis beyond this date, please submit an application for continuation approval to the ERC Secretariat by **December 21, 2012**. The regulations require continuing review even though the research activity may not have begun until sometime after the ERC approval.

Please note that any unanticipated problems resulting from the implementation of this study should be brought to the attention of the ERC. You are required to submit any proposed changes to this study to the SSC and ERC for review and approval prior to initiation and advise the ERC when the study is completed or discontinued.

Work on this project may begin.

Sincerely,

CHRISTINE WASUNNA,
FOR: SECRETARY,
KEMRI ETHICS REVIEW COMMITTEE