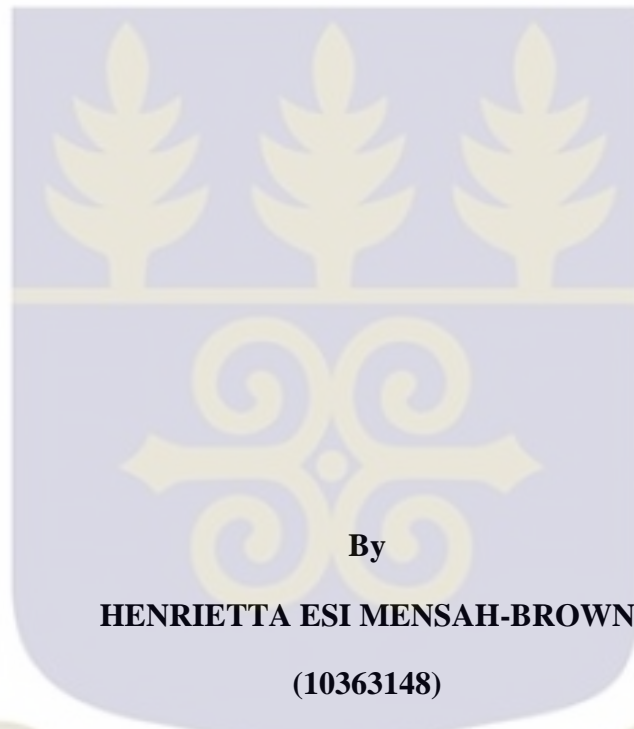**COLLEGE OF BASIC AND APPLIED SCIENCES**

UNIVERSITY OF GHANA

TARGETS AND PATTERNS OF ERYTHROCYTE INVASION INHIBITORY ANTIBODIES IN MALARIA

By

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WEST AFRICAN CENTRE FOR CELL BIOLOGY OF INFECTIOUS PATHOGENS

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Declaration

The experimental work presented in this thesis was done by me, Henrietta, E. Mensah-Brown, at the Department of Biochemistry, Cell and Molecular Biology and London School for Hygiene and Tropical Medicine under the supervision of Prof. Gordon A. Awandare (West African Centre for Cell Biology of Infectious Pathogens and Department of Biochemistry, Cell and Molecular Biology, University of Ghana) and Prof. David Conway (London School of Hygiene and Tropical Medicine).

All references have been duly cited.

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Henrietta E. Mensah-Brown (Student)

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Prof. Gordon A. Awandare (Supervisor)

………………………………………
Prof. David Conway (Co-Supervisor)

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Firstly, I would like to thank all participants and their guardians who gave their consent for sample collection, making the successful completion of this project possible.

My sincerest gratitude goes to my supervisor, Professor Gordon A. Awandare, for the opportunity to undertake this research in his laboratory, guiding me to perform all my experiments and enabling the successful completion of this thesis. I would like to thank Professor David Conway; whose inputs were invaluable. I appreciate the support of the members of the Infectious Disease Research Laboratory of the Department of Biochemistry, Cell and Molecular Biology. My special thanks go to Dr. James Abugri and Dr. Emmanuel Amlabu.

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I am also grateful to the staff of the Immunology Department of Noguchi Memorial Institute for Medical Research and the Department of Biochemistry, Cell and Molecular Biology for their indispensable help during my project. I thank my husband and my family for their support and prayers throughout this PhD.

Dedication

To God. Thank you for making all things beautiful in YOUR time.

To my husband, Rupert. I love you forever and a day.

In memory of Helena Ivy Chinbuah, for all that she was to me.

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List of abbreviations

ACTs	Artemisinin combination therapies
AMA-1	Apical membrane antigen-1
BCA	Bicinchoninic acid
CFDA-SE	5(6)-Carboxyfluorescein diacetate N-succinimidyl ester
CPM	Complete parasite medium
CR1	Complement receptor 1
CSP	Circumsporozoite protein
DBL	Duffy binding ligand
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
EIR	Entomological inoculation rate
EBA	Erythrocyte binding antigen
EBL	Erythrocyte binding ligand
FL	Full length ectodomain
GRA	Granulocytes
Hb	Haemoglobin

HbAA   Haemoglobin AA

HbAC   Haemoglobin AC

HbAS   Haemoglobin AS

HSPG   Heparan-sulphate proteoglycans

IgG   Immunoglobulin G

IRS   Indoor residual spraying

ITNs   Insecticide treated nets

IPTp   Intermittent preventive treatment in pregnancy

LLITNs   Long lasting insecticide treated nets

LYM   Lymphocytes

MCHC   Mean corpuscular haemoglobin concentration

MCV   Mean corpuscular volume

MON   Monocytes

MMA   Mild malarial anaemia

MSMA   Moderate to severe malarial anaemia

MSP   Merozoite surface protein

MSP-1   Merozoite surface protein 1

PLT   Platelets

PDW   Platelet distribution width

PfEMP-1   *P. falciparum* erythrocyte membrane 1 protein

PVM   Parasitophorous vacuolar membrane

PWM   Parasite wash medium

RBC   Red blood cell

RDW   Red cell distribution width

Rh   *P. falciparum* reticulocyte-like binding homologue

SDS-PAGE   Sodium dodecyl sulphate polyacrylamide gel electrophoresis

SEM   Standard error of the mean

SMA   Severe malarial anaemia

UM   Uncomplicated malaria

Outline of thesis

The results of this thesis are presented in 4 chapters consisting of manuscripts of three papers (one of which is published), and a general discussion of all the work presented. These chapters are preceded by a general introduction and a review of literature. The reference for the published journal article is listed below.

1. **Mensah-Brown, H. E.,** Abugri, J., Asante, K.P., Duah, D., Dosoo, D., Atuguba, F., Agongo, G., Conway D. J. & Awandare, G. A. (2017). Assessing the impact of differences in malaria transmission intensity on clinical and haematological indices in children with malaria. *Malaria journal, 16*, 96. doi:10.1186/s12936-017-1745-8

Abstract

Background

As malaria transmission continues to decline, a better understanding of how transmission intensity and patterns influence clinical presentation of disease and the acquisition of immunity to malaria is important for our understanding of the pathophysiology of malaria and the changes in immune responses. Erythrocyte invasion is a crucial step in the life cycle of *Plasmodium falciparum*. However, the targets of naturally acquired invasion inhibitory antibodies remain unclear. In this study, the differences in clinical and haematological presentation of disease, the predictors of anaemia severity in children with malaria, living in three ecologically distinct areas of Ghana, with different transmission intensities was determined. Patterns of invasion inhibitory antibodies were also assessed to explore the impact of transmission intensity on these parameters. Additionally, the relationship between antibody levels and functionality of antibodies to key invasion ligands were evaluated in adults living in a holoendemic area of Ghana.

Methods

Blood samples were taken from children between the ages of 2 and 14 years with confirmed malaria in hospitals in three areas with different transmission intensities (Kintampo>Navrongo>Accra). Whole blood was used to perform comprehensive analysis of parasitological, clinical and haematological variables, whilst socio-economic details were collected using a questionnaire. Plasma samples separated from the whole blood collected were tested by enzyme linked immunosorbent assays for antibodies to *P. falciparum* invasion antigens, including erythrocyte binding antigens (EBA) 175, EBA140, EBA181, and reticulocyte binding-like homologue (Rh) 2, Rh4 and Rh5.

To identify the targets of invasion inhibitory antibodies, plasma samples were collected from 50 male adults living in a high transmission area with no recent history of clinical malaria. Antibodies against EBA and Rh proteins were detected using enzyme linked immunosorbent assays (ELISA). Immunoglobulin (IgG) fractions were purified from the plasma and used in erythrocyte invasion assays.

Results

Severity of malarial anaemia was more pronounced in children living in areas of high malaria transmission compared to children living in low endemic areas. Sickle cell trait was protective against anaemia severity in Kintampo (P=0.016), although this association was not statistically significant in Accra (*P*=0.379) and) and Navrongo (*P*=0.529). Parasitaemia was not a significant predictor of haemoglobin level, after controlling for age and gender.

In addition, antibodies against invasion antigens were negatively correlated with parasitaemia, and increased in an age-dependent manner. Regression analysis showed that breadth of antibody reactivity was exposure and age-dependent.

Levels of antibodies to EBA and RH antigens generally correlated with invasion inhibitory activity and breadth of antibody reactivity was predictive of inhibitory activity of purified IgG (ρ= 0.437, *P*= 0.009).

Conclusions

Our findings demonstrate significant differences in the haematological presentation and severity of malaria among children residing in areas with different transmission intensities. Patterns of antibody responses against invasion proteins are both antigen and exposure

dependent. Growth inhibitory activity was significantly associated with antibody levels and

the number of different antigens recognized.

CHAPTER ONE

1.0 Introduction

Malaria is still a global health concern, despite concerted efforts to eliminate it. In 2016, there were 214 million reported cases of malaria which resulted in 445,000 deaths (World Health Organization., 2017). Over 90% of malaria morbidity and mortality occurs in Sub-Saharan Africa, with *falciparum* malaria accounting for the majority of deaths and cases. *P. vivax, P. malariae, P. ovale and P. knowlesi* also infect humans, but do not cause as much overall mortality compared to *P. falciparum*. *P. falciparum* mostly affects pregnant women and children below the age of five years.

More than half the world's population remains at risk of malaria (World Health Organization., 2017), making its elimination a priority for the WHO. Over the last decade, multiple strategies have been deployed to help reduce the burden of malaria. These include the use of vector control strategies such as long lasting insecticide treated nets and indoor residual spraying, as well as the use of artemisinin based combination therapies (ACTs) and intermittent preventive treatment in pregnancy (IPTp). These interventions have helped reduce malaria incidence significantly in endemic areas, and resulted in malaria elimination in some countries, including Singapore, Sri Lanka and Paraguay. As a result of the threat posed by the emergence of insecticide resistant vectors (Trape *et al.*, 2011) and artemisinin-resistant parasites (Dondorp *et al.*, 2009) to these successes, the WHO outlined a strategy for malaria elimination which included the development of a vaccine that can reduce disease severity and malaria-associated mortality (World Health Organization., 2015). However, vaccine development efforts face multiple challenges.

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Naturally acquired immunity to malaria is non-sterile, anti-disease immunity (Marsh and Kinyanjui, 2006) which is acquired slowly after multiple episodes of clinical disease. Antibodies, produced by B lymphocytes, play an integral role in antimalarial immunity, evident from experiments in which antibodies passively transferred from clinically immune adults to patients with clinical malaria were able to reduce parasitaemia and treat clinical symptoms (Cohen *et al.*, 1961, Sabchareon *et al.*, 1991). But antibodies to malaria antigens are short-lived, and require boosting over time, through either multiple clinical episodes or asymptomatic carriage of parasitaemia (revierwed in Hviid *et al.*, 2015). Also, the mechanism underlying the acquisition of immunity to malaria is not fully understood, and this is further compounded by the immune evasion strategies of the parasites. These strategies include antigenic variation, functional redundancy, antibody-dependent enhancement of infection and epitope masking (Rénia and Goh, 2016).

The asexual blood stages are important in the acquisition of immunity. Erythrocyte invasion, which occurs during the blood stage is a crucial multi-step process mediated by several receptor ligand interactions (Cowman and Crabb, 2006). Two families of parasite proteins have been shown to mediate these interactions: erythrocyte binding ligand (EBL) and reticulocyte-like binding homologue (Rh) protein. Members of the EBL family such as EBA-175, EBL-1 and EBA-140 are known to bind Glycophorins A, B and C respectively (Maier *et al.*, 2003, Mayer *et al.*, 2009, Orlandi *et al.*, 1992, Sim *et al.*, 1994), by binding to sialic acid molecules on the glycophorins. On the other hand, Rh4 and Rh5 bind to complement receptor 1 and basigin (Crosnier *et al.*, 2011, Spadafora *et al.*, 2010) respectively, in a sialic acid independent manner. Antibodies against multiple invasion antigens have been shown to inhibit invasion significantly, and predict protection against malaria (Chiu *et al.*, 2014, Chiu *et al.*, 2015, McCallum *et al.*, 2008, Osier *et al.*, 2014a,

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Persson *et al.*, 2008, Tran *et al.*, 2014). However, the functional redundancy exhibited by the parasite makes the identification of the targets of invasion inhibitory antibodies difficult.

Observational studies have suggested that level of malaria transmission influences the clinical presentation of disease (Snow *et al.*, 1997, Greenwood, 1997) and the acquisition of immunity to malaria (Fowkes *et al.*, 2016), which is especially important to study in the era of declining transmission intensity. The acquisition and breadth of immunity to malaria correlates with exposure and increases in an age-dependent manner (reviewed in Hviid *et al.*, 2015). Serum antibodies from malaria exposed individuals have been shown to inhibit invasion significantly and the inhibitory activity of these serum antibodies is influenced by the level of malaria transmission and endemicity (McCallum *et al.*, 2008). Studies in a high seasonal transmission area in Mali demonstrated that *P. falciparum*-specific B cell memory is acquired less efficiently compared to tetanus-specific B cell memory (Weiss *et al.*, 2010). On the other hand, individuals living in lower endemic settings show long-lasting memory B cell memory despite little or no exposure to the parasites (Nogaro *et al.*, 2011, Wipasa *et al.*, 2010). Long-lasting B cell memory was also reported in Swedish travellers who had clinical history of acute malaria, but lived in a non-endemic area without re-exposure (Ndungu *et al.*, 2013). The variation observed in *Plasmodium*-specific B cell phenotypes may suggest that transmission intensity and patterns may influence the acquisition and maintenance of B cell memory (Fowkes *et al.*, 2016, Ryg-Cornejo *et al.*, 2016). Understanding how transmission intensity and patterns influence the clinical and haematological presentation of disease and the patterns of important invasion inhibitory antibodies is fundamental to our understanding of the pathophysiology of malaria and the changes in immune responses, especially in the era of declining transmission intensity.

In this study, the differences in clinical and haematological presentation of disease, the predictors of disease severity and the patterns of invasion inhibitory antibodies in children with clinical malaria in three areas with different malaria transmission intensities were assessed to determine the impact of transmission intensity on these parameters.

1.1. Aims and Specific Objectives

Aim 1. To assess the impact of transmission intensity on clinical and haematological indices in Ghanaian children with malaria.

Hypothesis

Differences in transmission intensity affect demographic, socioeconomic, clinical and haematological factors associated with malaria in children

Rationale

Malaria control strategies implemented by the WHO have yielded significant results that have led to a substantial decline in malaria morbidity and mortality over the past decade and a half. As transmission intensity decreases, there is a shift in the vulnerable age group to from children below the age of five years to older children. Additionally, clinical manifestations of malaria appear to be significantly influenced by transmission intensity (Greenwood, 1997, Snow *et al.*, 1997), that has significant implications for malaria case management. A better understanding of the relationship between transmission intensity and the clinical manifestation of disease is required for the implementation of targeted malaria strategies for different transmission settings, malaria declines over time. In this study, Ghana was used a model to examine the effect of significant differences in malaria transmission on key clinical and haematological parameters in children with malaria as transmission intensity decreases.

Aim 2. To compare the levels and breadth of antibodies against merozoite antigens, in children with malaria in three areas with varying transmission intensity.

Hypothesis

Patterns and levels of antibodies to EBA and Rh protein in individuals living in areas with different transmission intensities would differ in an antigen and exposure dependent manner.

Rationale

EBA and Rh proteins are important proteins used in erythrocyte invasion by *Plasmodium falciparum (Cowman et al., 2017).* Due the importance of these antigens to the survival of the parasite, they have been identified as important targets of protective immunity, and potential vaccine targets(Richards *et al.*, 2013). It is generally known that antibodies to parasite antigens are acquired as a result of cumulative exposure(Doolan *et al.*, 2009). Other studies have suggested that whilst antibody responses may be antigen-specific, they are influenced by differing malaria transmission levels and study population characteristics. A study in Kenya suggested that patterns of antibody acquisition are antigen specific, regardless of different transmission settings, but recommended the need for a more comprehensive study involving multiple antigens (McCallum *et al.*, 2017). Therefore, the relationship between antibody levels to several merozoite antigens and transmission intensity is not well characterized. The current study was designed to examine the relationships between humoral immune responses to an expanded panel of antigens and transmission intensity, age and parasitaemia.

Aim 3: To identify the antibody reactivity patterns in adult plasma that correlate with growth inhibition of *P. falciparum in vitro*.

Hypothesis

Potent invasion inhibitory activity is achieved by antibodies against particular combinations of invasion ligands

Rationale

The importance of antibodies to immunity against malaria was first demonstrated by passive transfer experiments that showed the ability of immunoglobulins from malaria-exposed adults to clear parasitaemia and mitigate the symptoms associated with malaria (Cohen *et al*., 1961, Bouharoun-Tayoun *et al*., 1990). These experiments further indicated that purified immunoglobulins from adults may be useful in studying the targets of anti-malarial antibodies. Anti-malarial antibodies have since been shown to act using various mechanisms, including direct inhibition of parasite growth (Beeson *et al.*, 2016). Antibodies against EBA and Rh proteins are able inhibit parasite growth in vitro (Bustamante *et al*., 2013, Lopaticki *et al*., 2011), supporting the importance of these antigens as targets of immunity. Vaccine efficacy has been related to growth inhibitory activity in non-human primates (Singh *et al.*, 2006, Douglas *et al.*, 2015), though similar findings have not been observed in human. Furthermore, some studies have shown that growth inhibitory activity is correlated with protective immunity, though this finding has not been consistent (Crompton *et al.*, 2010b, Perraut *et al.*, 2005, Dent *et al.*, 2008, McCallum *et al.*, 2008).

The objective of our study was to advance knowledge on the breadth and dynamics of antibodies to key invasion related antigens, and examine the relationship between functional

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activity (i.e. growth inhibition) and the antibody reactivities to EBA and Rh antigens in semi-

immune adults living in an endemic area of Ghana.

CHAPTER TWO

2.0 Literature Review

2.1. The Burden of Malaria

Malaria is endemic in mainly sub-Saharan Africa, South America and parts of Asia (Figure 2.1), with the majority of cases and deaths occurring in sub-Saharan Africa. According to the World health organization (WHO), 90% of malaria cases and 91% of deaths attributed to malaria occur in sub-Saharan Africa. Approximately 70% of malaria mortality occurred in children below the age of 5 years (WHO Malaria report, 2017). In Ghana, it is estimated that malaria accounts for about 42.1% of all outpatient department (OPD) cases and 48.1% of all hospital admissions among children less than 5 years (Ghana Health Service., 2017).

The WHO concedes that current estimates are conservative, and hope to deploy better strategies of estimating malaria burden in 2018. Although current estimates of malaria burden are alarming, malaria transmission and cases have shown a steady decline over the last decade and a half. Between 2010 and 2016, the incidence of malaria declined by 18% globally, whilst malaria mortality was decreased by at least 25% in almost all WHO regions (World Health Organization., 2017). These recent successes in malaria control have mainly been attributed to use of LLITNs and IRS, as well as the use of artemisinin based combination therapies (ACTs).

Malaria is most endemic in sub-Saharan Africa, which is made up mostly of developing countries where poverty is rife. An examination of the global distribution of per-capita gross

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domestic product (GDP) indicates that malaria is correlated to poverty, and malaria-endemic countries also have lower rates of economic growth than non-endemic countries (Sachs and Malaney, 2002). Malaria has been estimated to cost Africa at least US$12 billion in lost GDP annually (Gallup and Sachs, 2001). In 2014, Businesses in Ghana lost US$6.58 million because of malaria, 90% of which were direct costs, affecting employee attendance and productivity (Nonvignon *et al.*, 2016).
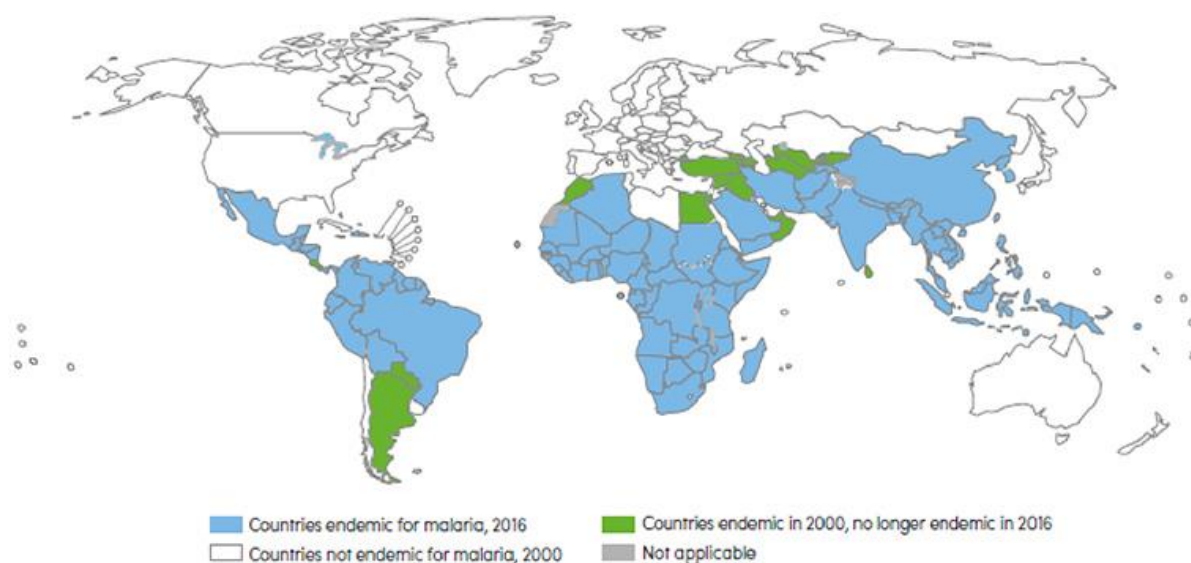
Figure 2.1. World Map of malaria endemicity, 2016. (World Health Organization., 2016)

2.2. The Life Cycle of *Plasmodium falciparum*

Malaria is caused by apicomplexan parasites of the genus *Plasmodium*. *Plasmodium falciparum* has a complex life cycle which occurs in the human host and a female *Anopheles* mosquito, which serves as the vector for malaria transmission. The most common vector for malaria transmission in Africa is the *Anopheles gambiae* species complex.

2.2.1. Liver Stage Infection

The life cycle begins when an infected female *Anopheles* mosquito takes a blood meal from a human, to acquire the nutritional protein needed to mature her eggs (Figure 2.2). The mosquito injects her proboscis into the skin, releasing an anticoagulant Factor Xa along with *Plasmodium* sporozoites into the blood stream of the human host (Stark and James, 1996). Using gliding motility, the sporozoites exit the dermis and migrate towards the liver upon entering the circulatory system. Upon entering the liver, the sporozoites adhere to highly sulphated heparin sulphate proteoglycans (HSPGs) found on hepatocytes, that activates calcium dependent protein kinase 6 (Coppi *et al.*, 2007). The subsequent signalling cascade that follows results in the activation/switch of sporozoites into invasive forms that can enter hepatocytes. Subsequently, the sporozoites begin a process called traversal, during which they traverse several hepatocytes before invading a single hepatocyte (Mota *et al.*, 2001). For hepatocyte invasion to occur, a sporozoite surface protein, circumsporozoite surface protein (CSP) must bind to HSPGs to activate the processing of CSP (Herrera *et al.*, 2015). Thereafter, several proteins, including apical membrane antigen-1 (AMA-1) and thrombospondin-related anonymous protein (TRAP), are involved in multiple steps which lead to invasion by the parasite and formation of the parasitiphorous vacuole. The sporozoites differentiate to form trophozoites, that subsequently become schizonts containing merozoites (Figure 2.2).
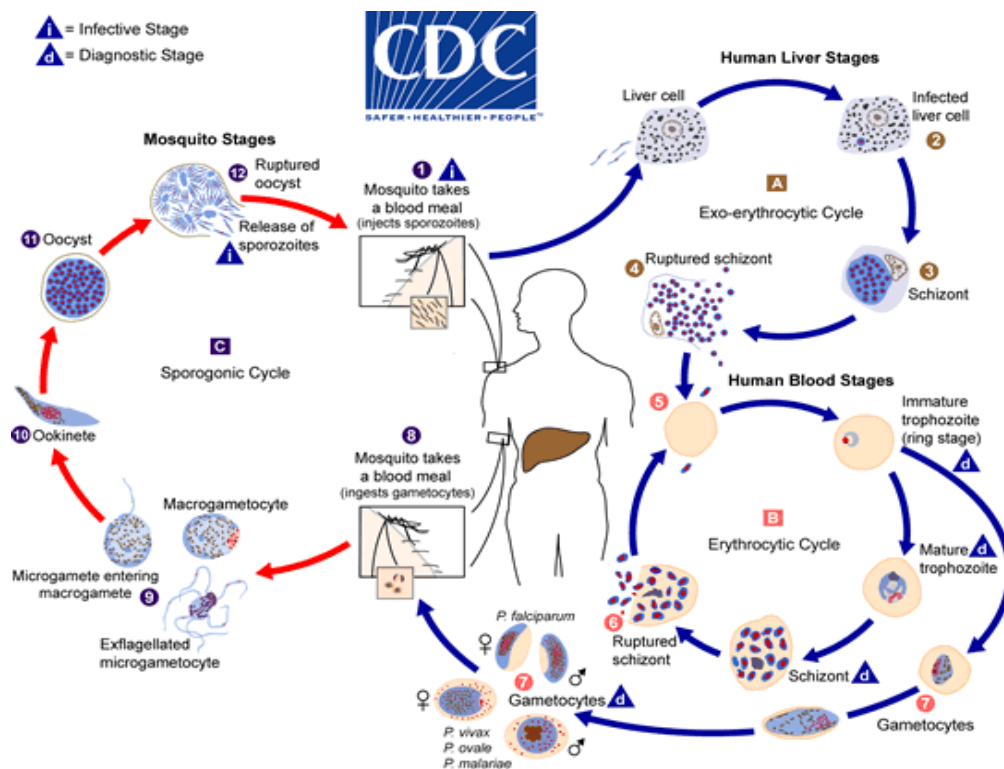
Figure 2.2. The life cycle of *P. falciparum*. When the female *Anopheles* bites a human host, sporozoites are injected into the bloodstream and invade hepatocytes. There, they differentiate into merozoites that invade erythrocytes and grow to form trophozoites and schizonts. Schizonts rupture to release merozoites that reinfect circulating erythrocytes. Some ring stage parasites differentiate into gametocytes beginning the sexual phase of the life cycle. Gametocytes are taken up into the gut of a feeding mosquito and mature to form male and female gametes. The fertilized zygote develops to an ookinete and an oocyst and finally sporozoites that migrate to the salivary glands to begin the cycle again (Source: Center for Disease Control., 2018).

The differentiation of sporozoites from trophozoites to schizonts usually takes approximately 6 days in falciparum malaria and leads to the formations of about 40,000 merozoites (Cowman *et al.*, 2016). Egress of merozoites begins with the breakdown of the parasitophorous vacuole membrane (PVM), degradation of mitochondria, cell detachment and formation of merosomes initiated by cysteine proteases of the serine repeat antigen

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(SERA) family (Sturm *et al.*, 2006). The merosomes, which consist of parasites enclosed in the host cell membrane, are released into the bloodstream by squeezing between two adjacent cells of the fenestrated endothelium of the liver sinusoid (Wirth and Pradel, 2012). The merosomes then travel through the bloodstream to the pulmonary capillary system where the parasites are injected into the bloodstream through a mechanism that is not yet fully understood (Baer *et al.*, 2007).

2.2.2. Asexual blood stages: the mechanism of erythrocyte invasion

Upon release from the hepatocytes into the blood stream, merozoites must undergo the process of erythrocyte invasion is a process involving multiple steps which occur swiftly (Figure 2.2). The mechanism of erythrocyte invasion can be divided into multiple phases that include initial attachment and deformation of the erythrocyte membrane; apical reorientation, tight junction formation and invasion; echinocytosis and recovery of infected red blood cell (Cowman *et al.*, 2016).

Initial attachment of the merozoite to the surface of the erythrocyte is thought to be mediated by a complex formed by a family of proteins known as merozoite surface proteins (MSPs) (Figure 2.3). Merozoite surface protein 1 (MSP1) forms multiple complexes with other MSPs, including MSP3 and MSP6 (Lin *et al.*, 2016). Inhibition of these complexes resulted in the inability of parasites to invade erythrocytes, signalling their possible importance in the invasion process (Lin *et al.*, 2016), though merozoites lacking MSP-1 can invade erythrocytes (Das *et al.*, 2015). The exact role of MSPs in the initial attachment remains unclear.

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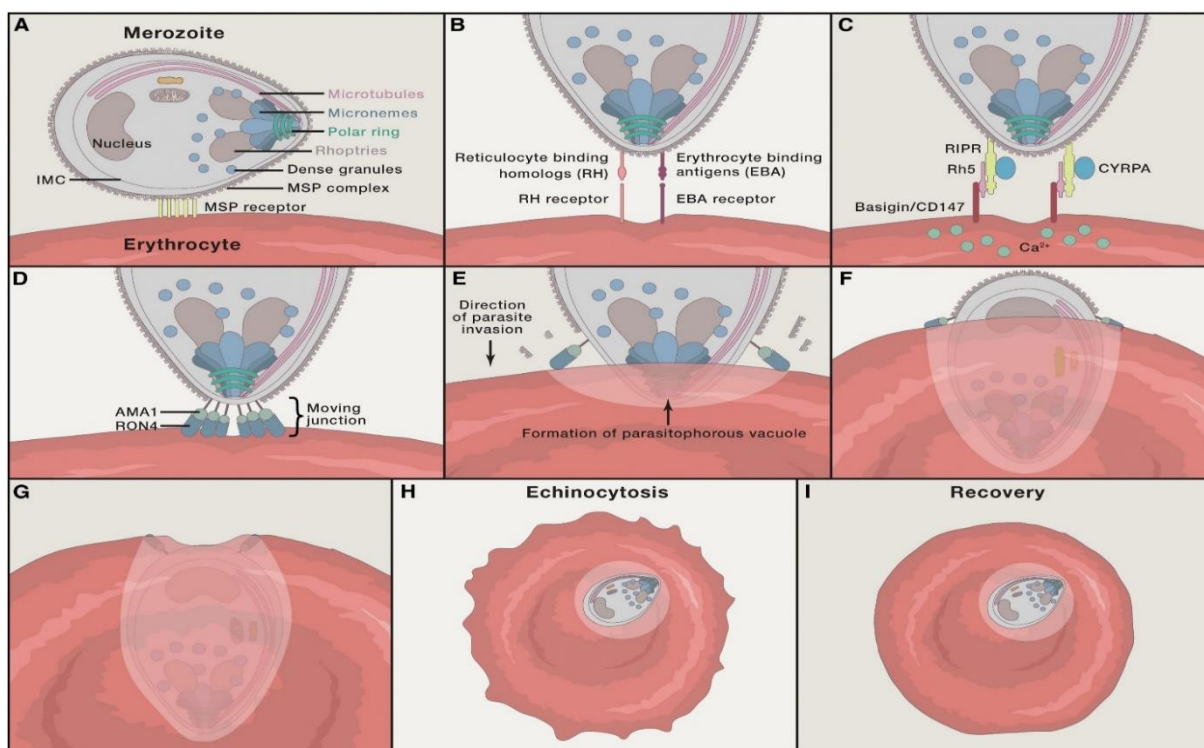
Figure 2.3. The process of merozoite invasion. The process of erythrocyte invasion begins with the initial contact of merozoite with the surface of the red blood cell. Initial attachment is mediated by merozoite surface proteins (MSPs) with their receptors (A). After initial attachment, the merozoite reorients such that the apical end is in contact with the red blood cell surface. Tight interactions mediated by proteins of the EBL and Rh family occur at this point (B). After these interactions, the Rh5 complex interacts with its receptor basigin, causing an influx of calcium and microneme secretion (C), which facilitates the insertion of the RON complex into the red blood cell surface and its interaction with AMA-1 (D). Using an internal merozoite actinomyosin complex, the merozoite invaginates itself into the red blood cell and a simultaneous release of rhoptry proteins leads to the formation of the parasitophorous vacuole (E-G). This is followed by echinocytosis (H) and recovery of the red blood cell (I). (Source: Cowman *et al.*, 2016).

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Subsequent to the initial attachment, a tight interaction occurs between the merozoite and erythrocyte occurs, that is mediated by two families of proteins: erythrocyte binding-like (EBL) proteins and the reticulocyte like binding homologue (Rh) proteins. Members of the EBL family include EBA-175, EBL-1 and EBA-140 that bind Glycophorins A, B and C respectively (Maier *et al.*, 2003, Mayer *et al.*, 2009, Orlandi *et al.*, 1992, Sim *et al.*, 1994). Rh4 and Rh5 bind to complement receptor 1 and basigin (Crosnier *et al.*, 2011, Spadafora *et al.*, 2010). Other members of the Rh family include Rh1, Rh2a and Rh2b bind to erythrocyte proteins that are not yet known. Overall, EBL and Rh proteins function similarly in the sequence of invasion, thus exhibiting functional redundancy. Initial attachment causes slight deformation of the erythrocyte, which becomes more pronounced following the engagement of the EBA and Rh proteins with their receptors. Calcineurin, in response to Ca^{2+} signalling initiated by initial attachment, facilitates ligation between EBAs/Rhs and host receptor, possibly by stabilizing EBA and Rh dimers (Figure 2.3). Subsequently, Rh5, in complex with PfRipr (Rh5-interacting protein), CyRPA (cysteine-rich-protective antigen) and possibly P113 (Chen *et al.*, 2011, Reddy *et al.*, 2015, Galaway *et al.*, 2017), bind to basigin to facilitate the reorientation of the merozoite such that the apical end is in direct contact with the erythrocyte surface. The interaction between the PfRh5 complex and its host receptor basigin is an essential step in erythrocyte invasion (Crosnier *et al.*, 2011), and is associated with the influx of Ca^{2+} ions into the host cell (Figure 2.3).

Apical reorientation is followed by the formation of a tight junction between apical membrane antigen 1(AMA-1) and the RON complex, consisting of RON2, RON3, RON4 and RON5 (Besteiro *et al.*, 2011) (Figure 2.3). RON2 serves as an anchor for the RON complex on the erythrocyte membrane. Tight junction formation is particularly interesting, since all proteins involved are parasite derived, with the parasite providing both the receptor

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and ligand for this interaction. The merozoite is propelled into the erythrocyte by the action of an actinomyosin motor, forming a parasitophorous vacuole membrane (PVM) around itself using part of the erythrocyte membrane and lipids expelled from its own secretory organelles (Ward *et al.*, 1993, Riglar *et al.*, 2011), and this serves as a membrane through which nutrients are exchanged between the parasite and the host cell. Echinocytosis, which involves the shrinkage of the erythrocyte and formation of protrusions on the erythrocyte surface, immediately follows the entry of the merozoite into the erythrocyte. This is thought to be caused by an influx of Ca^{2+} ions which occurs when the PfRh5 complex interacts with its host receptor basigin (Weiss *et al.*, 2015).

As the parasite develops from the ring stage into the trophozoite stage, its metabolism increases, and it forms a specialized organ called the food vacuole. The food vacuole is highly acidic and contains several proteases for the digestion of haemoglobin. The parasite digests about 60-80% of erythrocytic haemoglobin, deriving amino acids from the globin chain (Krugliak *et al.*, 2002). The proteases falcipain 2, plasmepsin II, plasmepsin IV, histo aspartic protease (HAP) and *Plasmodium* haem degradation protein (HDP) form a complex for the digestion of globin and the degradation of haem to haemozoin (Chugh *et al.*, 2013). The trophozoite undergoes deoxyribonucleic acid (DNA) replication and forms a schizont which divides into 16-32 merozoites. The merozoites then egress, to invade new erythrocytes.

Merozoites egress from erythrocytes in a multistep process involving the destabilization of the host cell cytoskeleton and breakdown of the PVM (Wirth and Pradel, 2012). This is followed by the host cell membrane turning inside out and rapidly vesiculating to release the merozoites. Glycosylphosphatidylinositol (GPI)-anchored proteins on the surface of the

merozoite, such as merozoite surface protein-1 (MSP-1), are processed by subtilisin 1 (PfSUB1) and subtilisin 2 (PfSUB2), to enable binding to the erythrocyte membrane protein spectrin.

2.2.3. Sexual Stage of *Plasmodium falciparum* Life Cycle

After multiple cycles of the asexual blood stage, some ring stage parasites differentiate to form gametocytes i.e. male and female sexual stages for transmission to the mosquito vector. The molecular mechanism of switching to gametocytogenesis is not fully understood. However, it is clear that merozoites are committed to either continue as asexual parasites or make the switch to gametocytes before egress. This is evidenced by the fact that all daughter merozoites from a single schizont all progress to form gametocytes of the same sex (Bruce *et al.*, 1990, Silvestrini *et al.*, 2000, Smith *et al.*, 2000). Multiple factors such as high parasitaemia, drugs such as fansidar and sulphadoxine, haemolysis and high reticulocyte count appear to trigger or enhance the formation of gametocytes (reviewed by Ngwa *et al.*, 2016). Recent studies have shown that AP2-G, a transcription factor epigenetically regulated by *P. falciparum* heterochromatin protein (Brancucci *et al.*, 2014) and *P. falciparum* histone deacetylase 2 (Coleman *et al.*, 2014) initiates a transcription cascade, resulting in commitment to gametocytogenesis (Josling and Llinás, 2015, Coleman *et al.*, 2014, Sinha *et al.*, 2014).

The gametocyte undergoes five stages of maturation before transmission to the mosquito. These stages sequester in the bone marrow and spleen, a strategy used to evade the host immune system. Upon maturation, stage V gametocytes re-enter the circulatory system, awaiting uptake by a female *Anopheles* mosquito during a blood meal (Figure 2.2).

The gametocytes are activated by the drop in temperature, change in pH and contact with xanthurenic acid when moved from a mammalian host to the midgut of the mosquito (Wirth and Pradel, 2012). The gametocytes egress from the erythrocyte through the rupture of the PVM and subsequent rupture of the erythrocyte membrane (Sologub *et al.*, 2011, Torres *et al.*, 2005). Male gametes then undergo, a process called ex-flagellation, which leads to the production of eight microgametes (Guinet *et al.*, 1996). The female gamete forms a large macrogamete that is fertilized by one of the male microgametes to form diploid zygote in the midgut of the mosquito. The zygote undergoes a single round of DNA replication, before forming a motile ookinete that crosses into the midgut epithelium (Figure 2.2). The parasite settles between the basal epithelial surface and the basal lamina where it develops into an oocyst after meiosis. The oocyst attaches to the midgut epithelium and stays there for about 10 days whilst it undergoes mitosis to form haploid sporozoites (Figure 2.2). The sporozoites are then released from the oocyst into the haemolymph circulation of the mosquito and travel to the salivary gland where they invade it. When the infected female *Anopheles* mosquito takes a blood meal, it injects the sporozoites into the vertebrate host and the cycle begins once more (reviewed by Smith *et al.*, 2014) (Figure 2.2).

2.3. Pathogenesis of Malaria

The asexual blood stages of malaria are responsible for the clinical presentation of disease which is observed when individuals have malaria. The majority of malaria cases reported are uncomplicated with symptoms such as fever, headaches, and vomiting, and usually resolve with treatment with ACTs. The more severe cases of malaria that cause morbidity present in three forms; namely severe malarial anaemia (SMA), and cerebral malaria (CM) that occur in mainly children, and placental malaria (also known as pregnancy-associated malaria) which occurs in pregnant women. The clinical manifestation of the severe forms

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of malaria is dependent on transmission intensity. Below we discuss the pathogenesis of each of the severe forms of malaria.

2.3.1. Pathogenesis of Severe Malarial Anaemia

SMA is the most common manifestation of life-threatening malaria in areas of high malaria transmission. The World Health Organization defines SMA as haemoglobin concentration below 5g/dL, with any density of parasitaemia (World Health Organization., 2000). The asexual stage of the life cycle of *P. falciparum* entails a repetitive cycle of invasion, rapture and reinvasion of erythrocytes. As the cycle progresses, the repeated cycles of erythrocyte lysis lead to anaemia. However, severity of the resultant anaemia is not correlated directly with parasitaemia, indicating that other factors may account for the severity of malarial anaemia. Etiological factors that lead to severe malaria include splenic sequestration of erythrocytes, dyserythropoiesis and bone marrow suppression; and immune mediated lysis of both infected and uninfected erythrocytes (reviewed in Perkins *et al.*, 2011) .

2.3.2. Pathogenesis of Cerebral Malaria

The World Health Organization defines CM as unexplained coma in a patient that has malaria with any parasite density (World Health Organization., 2000). Severe manifestations of malaria are caused by a phenomenon called parasite sequestration (Grau and Craig, 2012), which enables parasites to avoid the host immune system. CM is caused primarily by adhesion of infected erythrocytes to the vascular epithelial tissue of the brain. As infected erythrocytes mature from the ring stage to the young trophozoites, they adhere to human cells within the micro vascular circulatory system (Autino *et al.*, 2012). Cytoadherence by parasites is mediated by *Plasmodium falciparum* erythrocyte membrane protein-1 (PfEMP-1) which is expressed on the surface of trophozoite and schizont infected erythrocytes and

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encoded for by the *var* genes (reviewed in Hviid and Jensen, 2015). The extracellular portion of PfEMP-1 contains multiple adhesion domains that bind to host receptors such as ICAM-1 (Newbold *et al.*, 1997, Ochola *et al.*, 2011, Turner *et al.*, 1994) and endothelial progenitor cell receptor (EPCR) (Turner *et al.*, 2013), causing vaso-occlusion and inflammation.

2.3.3. Placental Malaria (PM)

Individuals living in an endemic area acquire immunity over time, and are often protected against clinical disease. However, during pregnancy, women become susceptible to placental malaria, which is caused by the sequestration of infected erythrocytes in the placenta. Sequestration is mediated by a parasite protein VAR2CSA (Salanti *et al.*, 2004), a member of the PfEMP-1 family, expressed on the surface of iRBCs which binds to chondritin sulphate A on the host membrane (Fried and Duffy, 1996). Placental sequestration causes inflammation which leads to malarial anaemia and low birth weight (Rogerson *et al.*, 2018).

2.4. Malaria Immunity

The acquisition of immunity to malaria is not yet fully understood, but is thought to be influenced by many factors including transmission intensity, host factors and parasite factors. The extent and frequency of exposure to malaria has been shown to be one of the key factors driving the acquisition of immunity. Children living in meso-to-hyperendemic areas acquire immunity against malaria at an earlier age with the highest disease burden among children below the age of five (Snow *et al.*, 1997). On the other hand, in low endemic areas, the burden of disease shifts, and the severe forms of malaria continues to occur in older children and adults (Snow *et al.*, 1997).

Immunity to malaria, however, is non-sterile anti-disease immunity which protects individuals from disease (Marsh and Kinyanjui, 2006) but not necessarily infection. Also, sustained contact with the parasite seems essential for the maintenance of protective immunity (reviewed in Hviid *et al.*, 2015). Asymptomatic children with detectable parasitaemia living in endemic areas are less likely to develop clinical disease compared to children without parasitaemia (Crompton *et al.*, 2008, Males *et al.*, 2008). Again, individuals who have acquired clinical immunity to malaria, but leave endemic areas for extended periods of time tend to lose their protective immunity, and become susceptible to infection once more (reviewed in Struik and Riley, 2004). Though there is evidence to suggest that all stages of the parasite elicit immune responses, naturally acquired immunity to malaria is targeted mainly at the asexual blood stages, where clinical symptoms occur.

Antibodies play an essential role in naturally acquired immunity to malaria. Studies have shown that passive transfer of immunoglobulins from clinically immune adults could reduce parasitaemia and treat symptoms in patients with clinical malaria (Cohen *et al.*, 1961, Sabchareon *et al.*, 1991). Multiple studies have shown that antibodies against several merozoite antigens are able to inhibit parasite growth *in vitro* (Reiling *et al.*, 2012, Chiu *et al.*, 2014, Chiu *et al.*, 2015, Triglia *et al.*, 2011, Tran *et al.*, 2014), inhibit erythrocyte binding by merozoites (Irani *et al.*, 2015), fix complement (Boyle *et al.*, 2015) and opsonize merozoites (Osier *et al.*, 2014a, Hill *et al.*, 2013, Joos *et al.*, 2010), emphasizing the key role of merozoite antigens in the acquisition of humoral immunity and protection against malaria. The magnitude and breadth of antibody responses are critical in NAI as individuals with a wider antibody response have a lower risk of developing clinical disease (Crompton *et al.*, 2010a, Osier *et al.*, 2008).

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2.5. Malaria Control Strategies

Transmission and incidence of malaria have been on a steady decline since 2000, and this has been attributed to vector control strategies and the introduction of ACTs for both chemo-preventive and chemotherapeutic uses.

In 2015, the WHO launched a global strategy for malaria in order to sustain current reductions in transmission and incidence, and push for elimination in all malaria endemic areas by 2030. The strategy outlined pillars which include the expansion of access to chemo-preventive and chemotherapeutic tools, the development of new tools to combat emerging drug-resistant parasites and insecticide resistant vectors, and the production of a vaccine with a protective efficacy of at least 75% (World Health Organization., 2015). The sections below highlight these strategies and their challenges as well as the current state of malaria vaccine development.

2.5.1. Vector control strategies

Vector control is an integral component of the control of malaria today. Eradication of malaria in various parts of the world was achieved through vector elimination. The WHO currently has two major strategies to control the mosquito vector, namely indoor residual spraying (IRS) and the use of long-lasting insecticide treated nets (LLITNs). LLITNS have proved to be even more widely successful than IRS, since they are more sustainable financially and in terms of logistics. LLITNs are impregnated with pyrethroids, which have low mammalian toxicity and last for about 3-4 years. Randomized controlled trials in Kenya, Ghana, The Gambia and Burkina Faso have demonstrated that wide-scale use of ITNs can reduce child mortality by approximately 20%, saving an average of six lives for every 1000 children aged 1–59 months protected every year (Lengeler, 2004). Both LLITNs and IRS

are dependent on one class of insecticides, pyrethroids. Pyrethroid resistance has been reported in most endemic countries (Trape *et al.*, 2011).

2.5.2. Therapeutic Drugs and Drug Resistance

Currently, the WHO recommends the use of ACTs as first line treatment for uncomplicated malaria in endemic areas. The common ACTs used include artemether-lumefantrine, artemether-amodiaquine, and dihydroartemisinin piperaquine. In 2009, the emergence of artemisinin resistant parasites was reported in Western Cambodia, in south east Asia, a known hotspot for drug resistance. Since the first reports, artemisinin resistant parasites have also been reported in Thailand (Putaporntip *et al.*, 2016, Talundzic *et al.*, 2015), China (Wang *et al.*, 2015), Myanmar (Feng *et al.*, 2015, Tun *et al.*, 2015) and Bangladesh (Mohon *et al.*, 2014).

Artemisinin resistance is characterized by slow clearance of the parasites, which causes treatment failure when a three-day treatment course is taken. This phenotype has been associated with a single nucleotide polymorphism in the *kelch13* propeller gene of the parasite (Ariey *et al.*, 2014).

2.5.3. Vaccine Development

The emergence of mosquitoes and parasites resistant to the current control strategies in use indicate that these strategies may not be enough to attain global elimination of malaria. The development of an effective vaccine against malaria that would provide protective immunity against malaria is key to the malaria elimination agenda.

Current vaccine development efforts are targeted at three stages of the parasite. These are the pre-erythrocytic stage (anti-infection vaccines), the asexual blood stages (anti-disease vaccines and the sexual stages (transmission blocking vaccines) of the parasites (Figure 2.4).

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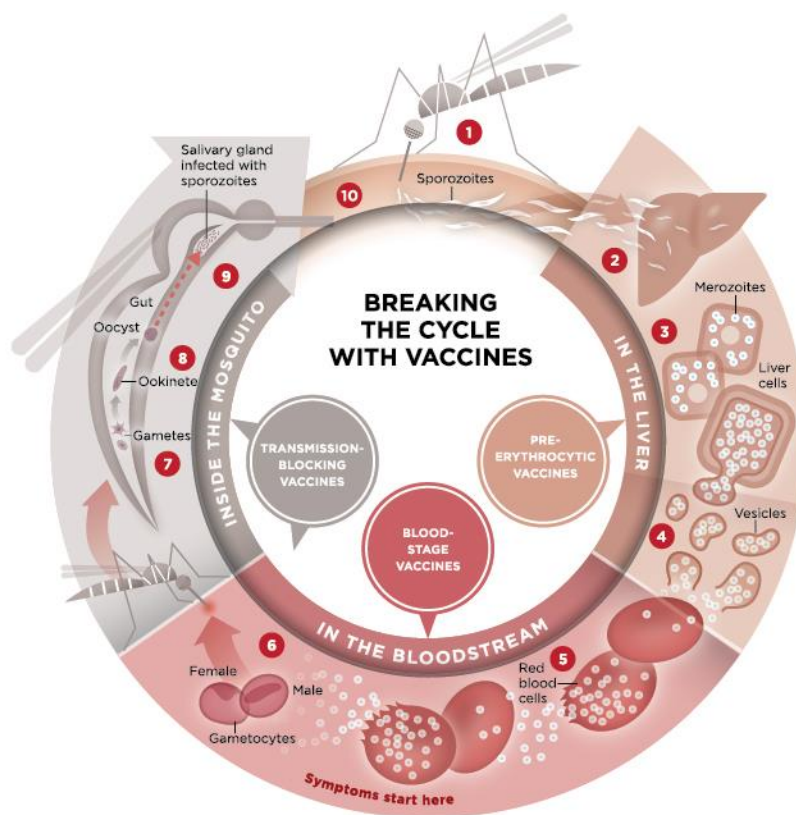
Figure 2.4. The life cycle of *Plasmodium* and the stages targeted for vaccine development (PATH Malaria Vaccine Initiative., 2018). The female *Anopheles* bites a human host, injecting sporozoites into the bloodstream. The sporozoites migrate to the liver where they invade hepatocytes, and differentiate into merozoites. The merozoites are released into the blood stream where they begin a cycle of invasion and replication in red blood cells. Some asexual stage parasites differentiate into gametocytes, that are taken up into the gut of a feeding mosquito and mature to form male and female gametes. The fertilized zygote develops to an ookinete and an oocyst and finally sporozoites that migrate to the salivary glands to begin the cycle again. The life cycle of the parasites provides an opportunity to break the cycle with pre-erythrocytic vaccines, blood stage vaccines and transmission blocking vaccines.

Malaria vaccine development is fraught with many challenges. Sequencing of the *Plasmodium* genome revealed that the parasite has 5300 genes (Gardner *et al.*, 2002). Approximately 60% of the *Plasmodium* proteome are hypothetical proteins with unknown functions. This is a major obstacle in the identification of the most important targets of protective immunity.

Another major challenge is antigenic variation. Protective antibody responses to malaria largely rely on antibodies to proteins which are highly polymorphic and exhibit antigenic variation such as PfEMP-1. PfEMP-1 is encoded for by the *var* genes (Baruch *et al.*, 1995, Smith *et al.*, 1995, Su *et al.*, 1995). Clonal antigenic variation exhibited in PfEMP-1 ensures that only a single variant of approximately 60 *var* genes are expressed at any one time, with the parasite showing the ability to switch from one *var* gene to another (reviewed in Hviid and Jensen, 2015). Another surface antigen AMA-1, is highly polymorphic with over 200 single nucleotide polymorphisms (Takala and Plowe, 2009). The production of a single component strain transcending vaccine seems unlikely, due to the complexity and diversity exhibited by the parasite.

The most advanced vaccine candidate, RTS,S/AS01, commercially known as Mosquirix, produced by Glaxo Smith Kline, targets the pre-erythrocytic stage of the life cycle of *P. falciparum.* It is a subunit vaccine containing circumsporozoite protein (CSP) and Hepatitis B surface antigen (HBsAg) formulated with an adjuvant AS01. RTS,S/AS01 has an efficacy of 20-50% against clinical and severe malaria (Agnandji *et al.*, 2012). Clinical trials for Mosquirix in Ghana are set to begin in 2018, where children between the ages of 6 months to 2 years would be vaccinated as part of the expanded programme for immunization (EPI) (Gallagher, 2017, PATH Malaria Vaccine Initiative., 2017).

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There are currently no blood stage or transmission blocking vaccines. However, Rh5, that is involved in erythrocyte invasion, has shown great promise as a candidate for blood stage vaccine development. Rh5 has been shown to elicit strain-transcending antibodies with the ability to neutralize merozoites and inhibit erythrocyte invasion (Douglas *et al.*, 2011, Reddy *et al.*, 2014).

The most advanced blood-stage vaccine candidate is GMZ2, which consists of the non-repeat region of *P. falciparum* glutamine rich protein (GLURP), genetically fused to a fragment of MSP3. In recent Phase 2b trials conducted in Burkina Faso, Ghana, Gabon and Uganda, vaccine efficacy was 11.3%, indicating that the formula may need to be improved to offer better protection (Sirima *et al.*, 2016).

2.6. Patterns of EBA and Rh antibodies

EBA and Rh proteins are important proteins used in erythrocyte invasion by *Plasmodium falciparum* (Cowman et al., 2017). Due to their widespread immunogenicity and functional roles in erythrocyte invasion, EBA and Rh ligands are promising vaccine candidates (Richards *et al.*, 2013).

Studies in West Africa have shown that there is significant variation in the expression of EBAs and Rh ligands in clinical isolates (Bowyer *et al.*, 2015, Mensah-Brown *et al.*, 2015). Tijani and colleagues showed that acquired invasion inhibitory antibodies to the EBAs switch to other targets over time (Tijani *et al.*, 2017). Taken together, these findings suggest that the immune response may be adapting to the diversity of invasion phenotypes and variable gene expression of the parasite. Hence the pattern of antibody responses to EBA and Rh antigens may differ between endemic areas.

A comparative study by Ford et al., showed that the antibody levels against EBA-175, EBA-140 and EBA-181 were much lower in individuals living in a hypoendemic area of Brazil compared to individuals living in a hyperendemic area of Cameroon (Ford *et al.*, 2007). Another study assayed levels to EBA-175 RII in two different endemic settings in Senegal, but did not directly compare levels between the two populations (Badiane *et al.*, 2013). In the case of Rh antigens, several studies have been conducted in several populations. However, it is difficult to directly compare antibody levels across areas of different endemicities, due to differences in methodology as well as the antigens used.

CHAPTER THREE

3.0 Paper 1: Assessing the impact of decreasing malaria transmission on clinical and haematological indices in children with malaria (Published in *Malaria journal (2017), 16*, 96. doi:10.1186/s12936-017-1745-8)

3.1. Abstract

Background: Malaria control interventions have led to a decline in transmission intensity in many endemic areas, and resulted in elimination in some countries. This decline however, will lead to delayed acquisition of protective immunity and thus impact disease manifestation and outcomes. Therefore, we assessed the variation in clinical and haematological parameters in children with malaria across three areas in Ghana with varying transmission intensities.

Methods: A total of 549 children between the ages of 2 and 14 years with confirmed malaria were recruited in hospitals in three areas with varying transmission intensities (Kintampo>Navrongo>Accra) and a comprehensive analysis of parasitological, clinical, haematological and socio-economic parameters was performed.

Results: Areas of lower malaria transmission tended to have lower disease severity in children with malaria, characterized by lower parasitaemias, and higher haemoglobin levels. In addition, total white cell counts and percent lymphocytes decreased with decreasing transmission intensity. The heterozygous sickle haemoglobin genotype was protective against disease severity in Kintampo (P=0.016), although this relationship was not statistically significant in Accra (*P*=0.379) and) and Navrongo (*P*=0.529). Parasitaemia levels were not a predictor of haemoglobin level, after controlling for age and gender.

However, higher haemoglobin levels in children were associated with having fathers who had any type of employment (P<0.05) and mothers who were teachers (P<0.05).

Conclusions: Our findings demonstrate significant differences in the haematological presentation and severity of malaria among areas with different transmission intensities in Ghana, indicating that these factors need to be considered in planning the management of the disease as the endemicity is expected to decline after control interventions.

3.2. Introduction

The World Health Organization estimates that malaria still causes approximately 212 million cases annually worldwide, with 429, 000 deaths, mainly in children below the age of 5 years and pregnant women (World Health Organization., 2016). The most severe forms of the disease are caused by *Plasmodium falciparum,* which accounts for more than 90% of malaria cases globally(World Health Organization., 2016). The commonest life-threatening forms of malaria in children are severe malarial anaemia (SMA) and cerebral malaria (CM). As such, malaria is a leading cause of anaemia in children in endemic areas (Newton *et al.*, 1997), including Ghana (VanBuskirk *et al.*, 2014), and SMA contributes to over 50% of malaria-related deaths in holoendemic areas (Obonyo *et al.*, 2007).

The use of vector control strategies such as long-lasting insecticide treated nets (LLITNs) and indoor residual spraying (IRS), combined with the use of the efficacious artemisinin combination therapy (ACT), have significantly decreased malaria transmission. This has led to a greater than 50% decline in malaria-related mortality in the last decade, from over a million deaths to under 500,000 annually (Malaria, 2005, World Health Organization., 2016). Since clinical manifestations of malaria vary with differences in transmission levels (Greenwood, 1997, Snow *et al.*, 1997), it is likely that the decreasing transmission intensities will be accompanied by significant changes in clinical and haematological indicators of anaemia severity. Studies in north-eastern Tanzania have indicated that decreasing levels of malaria transmission result in an increase in the median age of children with malaria, a reduction in the incidence of malaria and an increase in case fatality (Reyburn *et al.*, 2005). Similar findings in the Gambia and Kenya confirmed that average age and risk of fatal disease is highest among children living in low endemic areas, in comparison to areas of high endemicity (Snow *et al.*, 1997). Therefore, as many countries deploy malaria elimination

strategies, a comprehensive analysis of the impact of decreasing malaria transmission on clinical and haematological indicators of disease is necessary to inform appropriate management of a changing malaria phenotype.

Given the significant effects of common haemoglobinopathies on malaria severity, a comprehensive study of clinical and haematological parameters must include an investigation of the role of these genetic factors. Haemoglobin S (HbS) and haemoglobin C (HbC) variants are common in malaria-endemic populations, with more than a fourth of the African population carrying one variant or the other (Flint *et al.*, 1998). Heterozygous carriers of HbS (i.e. sickle cell trait, HbAS) are protected against severe and fatal forms of malaria (Friedman, 1978, Williams *et al.*, 2005b). This protection is thought to be achieved by preventing the development of high density parasitaemia (Allen *et al.*, 1992, Lell *et al.*, 1999, Stirnadel *et al.*, 1999), and accelerating the acquisition of natural immunity to malaria in carriers (Williams *et al.*, 2005a). Studies conducted in Ghana (Amoako *et al.*, 2014) and elsewhere (Allen *et al.*, 1992, Kreuels *et al.*, 2010, Williams *et al.*, 2005b) have confirmed the protection conferred by sickle cell trait. However, the protective role of HbC remains unclear with some studies showing protective effects (Agarwal *et al.*, 2000, Gilles *et al.*, 1967, Modiano *et al.*, 2001), while others have found no evidence of protection against malaria (Guinet *et al.*, 1997). In Ghana, studies have shown that HbC protects against malaria, though to a lesser extent compared to HbS (Amoako *et al.*, 2014, Mockenhaupt *et al.*, 2004).

In this study, we took advantage of the significant differences in malaria transmission across ecological zones in Ghana, to analyse the changes in key clinical and haematological parameters in children with malaria as transmission intensity decreases. We also examined

the protective roles of HbC and HbS against severe malaria, amidst varying transmission

intensities across three endemic areas in Ghana.

3.3. Study Participants and Methods

3.3.1. Sample Collection

Study participants were recruited in hospitals in three ecologically distinct areas in Ghana, namely: the Ledzekuku Krowor municipality (LEKMA) in Accra; the Kassena and Nankana districts in Northern Ghana, and the Kintampo Municipality in the middle belt of Ghana. Malaria transmission intensity as measured by the entomological inoculation rates (EIR) are highest in Kintampo, followed by Navrongo, and lowest in Accra (Kintampo>Navrongo>Accra) (Dery *et al.*, 2010, Kasasa *et al.*, 2013, Klinkenberg *et al.*, 2008). A detailed description of the study sites is presented in our recent report (Mensah-Brown *et al.*, 2015).

Children between the ages of 2-14 years who had lived in the community for at least 6 months and presented with symptoms of malaria were screened for malaria with rapid diagnostic tests (RDTs) using a drop of blood from a finger prick. After obtaining informed consent from RDT-positive children, 0.5 mL of blood was collected into EDTA-coated vacutainer tubes (BD biosciences) for parasitological and haematological analysis.

3.3.2. Sample size considerations

Sample size projections were based what was required to adequately examine the relationship between antibody levels and transmission intensity (Aim 2). It was estimated that a sample size of 124 children was required to detect correlations with coefficient≥0.25 at >80% power and α=0.05 level. Therefore, samples were collected from at least 124 children at each site, which allowed analyses to be performed at two levels: at the zonal level, for which correlations and regression were performed for each distinct zone, and a meta-analysis encompassing all the zones to obtain a comprehensive country-wide profile.

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3.3.3. Determination of clinical and haematological indices

Haemoglobin levels, erythrocyte indices [red blood cell (RBC) counts, mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), and red cell distribution width (RDW)], leucocyte indices [total white blood cell (WBC) count, percent lymphocytes (LYM%), percent monocytes (MON %), percent granulocytes (GRA %), and platelet indices [platelet count (PLT), mean platelet volume (MPV), and platelet distribution width (PDW)] were analysed using an automated haematology analyser. Thick blood smears were prepared and stained with Giemsa and examined for parasites using oil immersion microscopy. Parasite density per µL of blood was determined by counting the number of parasites per 200 WBCs and multiplying by the total WBC count obtained from an automated haematology analyser.

3.3.4. Determination of haemoglobin types

Haemoglobin types were determined by Hb electrophoresis according to manufacturer's instructions (Helena Laboratories, Beaumont, TX, USA). Briefly, whole blood was haemolysed using haemolyse reagent containing 0.005 M EDTA with 0.01% potassium cyanide. Patient haemolysates and controls were then loaded on to cellulose acetate plates and electrophoresed at 350 V for 25 minutes in a Tris-EDTA/boric acid buffer (pH 8.4, ionic strength 0.035). Hb electrophorotypes were identified by comparing to control bands.

3.3.5. Classification of disease severity

Children recruited into this study were classified into three categories based on the severity of malarial anaemia. The categories were defined as: (1) Uncomplicated malaria (UM): Children with a malaria-positive smear for *P. falciparum* parasitaemia (of any density) without anaemia (i.e., Hb ≥ 11.0 g/dL); (2) Mild malarial anaemia (MMA): Children with a

malaria-positive smear for *P. falciparum* parasitaemia (of any density) with $8.0 \leq Hb < 11$ g/dL; (3) Moderate-to-severe malarial anaemia (MSMA): Children with a malaria-positive smear for *P. falciparum* parasitaemia (of any density) with $Hb < 8.0$ g/dL. For some analyses, we also classified participants according to parasite density, using $\geq 10,000$ parasites/µL as definition for high-density parasitaemia.

3.3.6. Statistical Analysis

Data were analysed using Minitab (version 17). Kolmogorov-Smirnov tests were used to determine normality of the data before application of further tests. Oneway ANOVA or Kruskal-Wallis tests were applied for across group comparisons for normally and non-normally distributed data respectively. Post-hoc tests of either student t-test or Mann-Whitney U tests were applied for pairwise analysis where necessary. Chi-square analysis was used to compare categorical variables and determine likelihood ratio for association. Univariate and multivariate regression analyses were carried out to determine the predictors of malaria severity. For all analyses, $P<0.05$ was considered statistically significant.

3.4. Results

3.4.1. Comparison of clinical and demographic parameters across transmission areas

To investigate the variation in clinical presentation of malaria as transmission reduces, we enrolled 568 malaria positive children aged between 2-14 years, who were exposed to different intensities of *P. falciparum* transmission (Kintampo>Navrongo>Accra). While there was no difference in the proportion of females recruited at each site (Table 3.1), children enrolled in Accra were generally older than children recruited in Navrongo (P<0.001) and Kintampo (P<0.001). In addition, haemoglobin levels were higher in the area of lowest transmission intensity, being significantly higher in children in Accra compared to Navrongo and Kintampo (P<0.001 for both comparisons; Table 1). Parasitaemia levels were higher in areas of higher transmission, with the Kintampo group having the highest parasitaemia (P<0.001 vs Navrongo and vs Accra). Parasitaemia levels in children in Navrongo were higher than in Accra, but this difference was not statistically significant. (P=0.103; Table 3.1). These data therefore, suggest that clinical presentation of malaria tends to be associated with lower parasitaemia and higher haemoglobin levels as transmission intensity reduces.

Table 3.1. Demographic and clinical characteristics of study participants

	Kintampo	Navrongo	Accra	Total	P Value
Number of enrolees	275	144	149	568	
Female, %	44.00	52.78	43.28	46.11	0.174*
Age, years	5.37 (0.20)	5.40 (0.40)	6.246 (0.280)	5.60 (0.16)	<0.001**
Haemoglobin levels, g/dL	9.83 (0.10)	9.95 (0.16)	10.72 (0.14)	10.10 (0.08)	<0.001**
Parasitaemia, parasites/μL	148, 435 (12, 714)	46,429 (3, 977)	37, 999 (3, 274)	101, 210 (10,056)	<0.001**
Body Temperature (^{0}C)	37.61 (0.07)	37.76 (0.17)	38.80 (0.09)	37.93 (0.07)	<0.001**

Relative malaria transmission intensities as measured by entomological inoculation rates in the areas are in the order Kintampo>Navrongo>Accra

Data for age, haemoglobin, and parasitaemia are presented as means (standard error of the mean, SEM).

*P-value obtained from Chi Square Test, **P-value obtained from ANOVA

3.4.2. Comparison of haematological parameters in children with malaria across transmission areas.

The associations between transmission intensity and haematological indices was also explored by comparing erythrocyte, leucocyte, and platelet indices in children with malaria across the study sites. RBC counts were significantly higher in areas of lower transmission, mirroring the pattern observed for haemoglobin levels (P<0.001 for both Accra vs Navrongo and Accra vs Kintampo; Table 2). However, MCV was lowest in the area of lowest transmission, with levels in children in Accra being significantly lower than those in Navrongo (P<0.001) and Kintampo (P<0.001; Table 3.2). MCHC and RDW did not appear to directly relate to transmission intensity, with both indices showing the highest levels in Navrongo (P<0.001 for across group comparisons for both indices, Table 3.2). Total WBC counts, as well as percentage lymphocytes in children with malaria decreased with decreasing transmission intensity across the three sites (P=0.029 and P<0.001, respectively, Table 3.2). Percentage monocytes was significantly lower in children in Navrongo, compared to children in Kintampo (P<0.001) and Accra (P<0.001), while percentage granulocytes was lowest in Kintampo, compared to children in Navrongo (P<0.001) and Accra (P<0.001). Platelet counts and PDW were both highest in Navrongo (P=0.008 and P<0.001 across groups, respectively, Table 3.2), suggesting no direct relationship with transmission intensity. MPV increased with decreasing malaria transmission levels, with children from Accra showing significantly higher levels relative to Navrongo (P<0.001) and Kintampo (P<0.001; Table 3.2).

Table 3.2. **Comparison of haematological indices in children with malaria across three transmission areas**

Haematological indices	Study Sites			Kruskal Wallis
	Kintampo (N=266) *median* (*IQR*)	Navrongo (N=144) *median* (*IQR*)	Accra (N= 117) *median* (*IQR*)	*P*-value
Erythrocyte Indices				
RBC (10^3/ mm^3)	4.0 (3.5-4.4)	4.1 (3.6-4.5)	4.4 (4.0-4.8)	<0.001
MCV (μm^3)	78.0 (74.0-81.0)	75.0 (70.0-78.0)	73.7 (68.5-77.7)	<0.001
MCH (pg.)	25.5 (23.6-26.7)	25.8 (23.9-27.3)	25.1 (23.1-26.9)	0.131
MCHC (g/dL)	32.4 (31.7-33.3)	34.3 (33.5-35.2)	34.1 (33.1-35.0)	<0.001
RDW (%)	15.1 (14.1-16.5)	16.4 (15.4-17.6)	14.7 (13.6-16.2)	<0.001
Leucocyte indices				
WBC (10^3/mm^3)	8.5 (6.6 -13.0)	8.2 (5.8-10.4)	7.5 (5.5-10.3)	0.016
LYM (%)	30.1 (21.0-41.3)	19.4 (13.2-28.9)	18.5 (13.8-29.7)	<0.001
MON (%)	6.8 (5.3-8.6)	5.5 (3.8-7.5)	7.1 (4.5-11.0)	<0.001
GRA (%)	62.6 (49.5-72.6)	75.2 (64.2-83.1)	72.4 (61.6 -79.7)	<0.001
Platelet Indices				
PLT (10^3/mm^3)	25.5 (87.8-182.0)	141.0 (100.0-210.3)	126.5 (78.3-210.3)	0.048
MPV (μm^3)	7.3 (6.8 -8.3)	7.8 (7.3-8.3)	9.3 (8.8-10.2)	<0.001
PDW (%)	15.1 (13.2-17.3)	14.7 (11.5-16.5)	11.9 (10.8-14.0)	<0.001

Data are presented as medians (first quartile-third quartile)

Relative malaria transmission intensities as measured by entomological inoculation rates in

the areas are in the order Kintampo>Navrongo>Accra

3.4.3. **Impact of haemoglobin S and C on clinical and haematological indices**

We investigated the impact of haemoglobins S and C on the clinical and haematological indicators of disease severity in the entire cohort of children with malaria. Haemoglobin levels differed between children with HbAS compared to HbAA genotype (*P=0.033).* However, there was no significant difference in haemoglobin levels in children with HbC genotypes relative to those with the HbAA genotype (*P*=0.975; Table 3.3). However, RBC counts (*P*=0.027) and RDW (*P*=0.002) were relatively higher in the HbAS and HbAC groups compared to the HbAA genotypes (Table 3.3). On the other hand, MCV (*P*=0.007) and MCH (*P*=0.008) levels were lower in the HbAS and HbAS groups relative to the HbAA group. Platelets counts were significantly higher in HbAS relative to HbAA (*P*=0.020), however, there was no difference between the HbAC and HbAA groups (*P*=0.567; Table 3.3). MPV levels were lower in the HbAS and HbAC groups relative to HbAA. The remaining indices were not significantly different across the groups (Table 3.3).

Additional analysis examined the relationship between haemoglobin genotypes and peripheral parasite densities in children recruited from the three study sites separately. These analyses showed a protective effect of HbAS on parasite density, however, this effect appeared to be site-specific (Figure 1). Parasite densities were significantly lower in children with HbAS compared to the HbAA group (*P*=0.016) in children from Kintampo, however, this relationship was not observed in the Accra and Navrongo cohorts (*P*=0.379 and *P*=0.529 respectively; Figure 3.1). These results suggest that the protective effect of sickle cell trait may be influenced by transmission intensity.

Table 3.3. Relationship between haemoglobin types and haematological indices in children with malaria

Haematological indices	Haemoglobin type			
	AA	AS	AC	
	(N= 346)	(N=46)	(N=37)	
	$median(IQR)$	$median(IQR)$	$median(IQR)$	P-value
Erythrocyte Indices				
Haemoglobin (g/dL)*	10.3 (9.0-11.4)	10.80 (9.4-11.65)	10.30 (8.57-11.55)	0.219[#]
RBC(10^3/ mm^3)	4.1 (3.7-4.5)	4.3 (3.9-4.6)	4.2 (3.6-4.8)	0.187
MCV (μm^3)	77.0 (73.0-80.0)	74.5 (71.8-77.9)	73.5 (68.7-79.0)	0.007
MCH (pg.)	25.6 (23.8-26.9)	24.5 (23.7-26.6)	24.7 (22.7-25.7)	0.008
MCHC (g/dL)	33.3 (32.2-34.4)	33.4 (32.2-34.4)	32.8 (32.1-34.3)	0.743
RDW (%)	15.4 (14.3-16.7)	16.2 (15.0-17.4)	15.6 (14.8-17.4)	0.002
Leucocyte indices				
WBC (10^3/mm^3)	8.2 (6.2-10.5)	8.9 (6.1-11.0)	9.0 (6.9-13.0)	0.058
LYM (%)	27.3 (17.1-40.5)	26.3 (17.4-41.3)	27.0 (15.9-44.0)	0.373
MON (%)	6.3 (4.8-8.6)	6.4 (5.1-8.5)	6.4 (5.1-8.3)	0.253
GRA (%)	64.5 (49.5-76.4)	63.7 (51.7-76.9)	62.7 (44.6-74.8)	0.226
Platelet Indices				
PLT (10^3/mm^3)	141 (92.0-211.0)	166.5 (106.0-231.3)	137.0 (79.0-234.0)	0.044
MPV (μm^3)	7.8 (7.3-8.5)	7.6 (6.9-8.1)	7.5 (7.1-8.5)	0.043
PDW (%)	14.6 (12.4-16.7)	14.4 (11.5-16.3)	14.4 (11.9-18.7)	0.555

Data are presented as medians (first quartile-third quartile)

* Sample sizes for haemoglobin levels were AA =431, AS =49 and AC =49

[#] *P*-value obtained from ANOVA.

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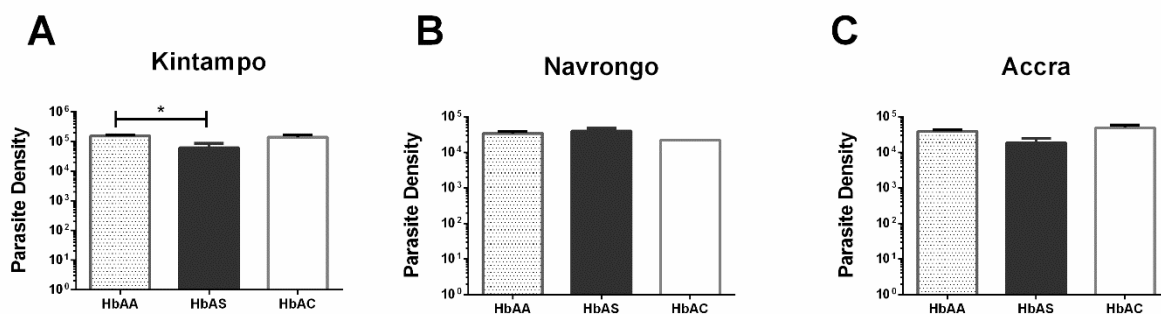
Figure 3.1. Impact of sickle cell trait on parasite density in children with malaria. Parasite densities in malaria positive children with HbAA, HbAS and HbAC haemoglobin genotypes in (A) Kintampo (B) Navrongo and (C) A were compared to determine the impact of sickle cell trait on high density parasitaemia infections. Statistical significance was ascertained by comparing each of the HbAS and HbAC groups with the HbAA group, using Mann-Whitney U test.

3.4.4. Determinants of malaria severity

Since disease severity in our cohort was generally characterized by anaemia, we stratified the children into three malaria categories based on haemoglobin levels: UM (Hb $\geq$ 11g/dL, n=194), MMA (8.0 $\leq$ Hb < 11 g/dL, n=279) and MSMA (Hb < 8.0g/dL n=65). The relationship between malaria severity and various demographic, clinical and haematological parameters was then examined by comparing across the malaria groups. Children with UM were significantly older than children with MMA (P<0.001) and MSMA (P<0.001; Figure 3.2A). Children with MSMA were also significantly younger than children with MMA (P=0.003; Figure 3.2A). Comparison across transmission areas revealed that lower transmission was associated with less severity of malarial anaemia, such that the proportion of patients from Accra who had MSMA was significantly lower than that observed in Navrongo (P=0.002) and Kintampo (P<0.001; Figure 3.2B). Conversely, the proportion of

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children with UM in the Accra group was significantly higher than the corresponding proportions in Navrongo (P=0.004) and Kintampo (P<0.001; Figure 3.2B). There were no significant differences in the proportions of children with MMA across the three sites (P=0.104).

The relationships of anaemia severity with parasite density and haemoglobin types was also investigated. There was no significant relationship between high density parasitaemia (≥10,000 parasites/L) and anaemia severity, which is consistent with previous reports [27] demonstrating that the two parameters were not directly related. There was however, evidence of the protective effects of HbAS, such that children with this genotype had a significantly lower proportion of MSMA (P=0.011) and a higher proportion of UM (P<0.001) compared to the HbAA group (Figure 3.2D). There were no significant differences in proportions of disease in the HbAC group compared to HbAA group (P=0.685, Figure 3.2D), suggesting that the protective effects were specific for HbAS only.

3.4.5. Relationship between haematological factors and malaria severity

We further compared haematological indices across the three categories to determine indicators of disease severity. With the exception of RDW, all red cell indices examined were lower in the malarial anaemia groups compared to UM (Table 4), which was expected since anaemia is primarily a red cell-related disease. In particular, RBC counts and MCV decreased with increasing anaemia severity across the groups (P<0.001 and P=0.003; Table 4). In addition, MCH levels were significantly lower in children with MMA (P<0.001) and MSMA (P<0.001) compared to the UM group P=0.002).
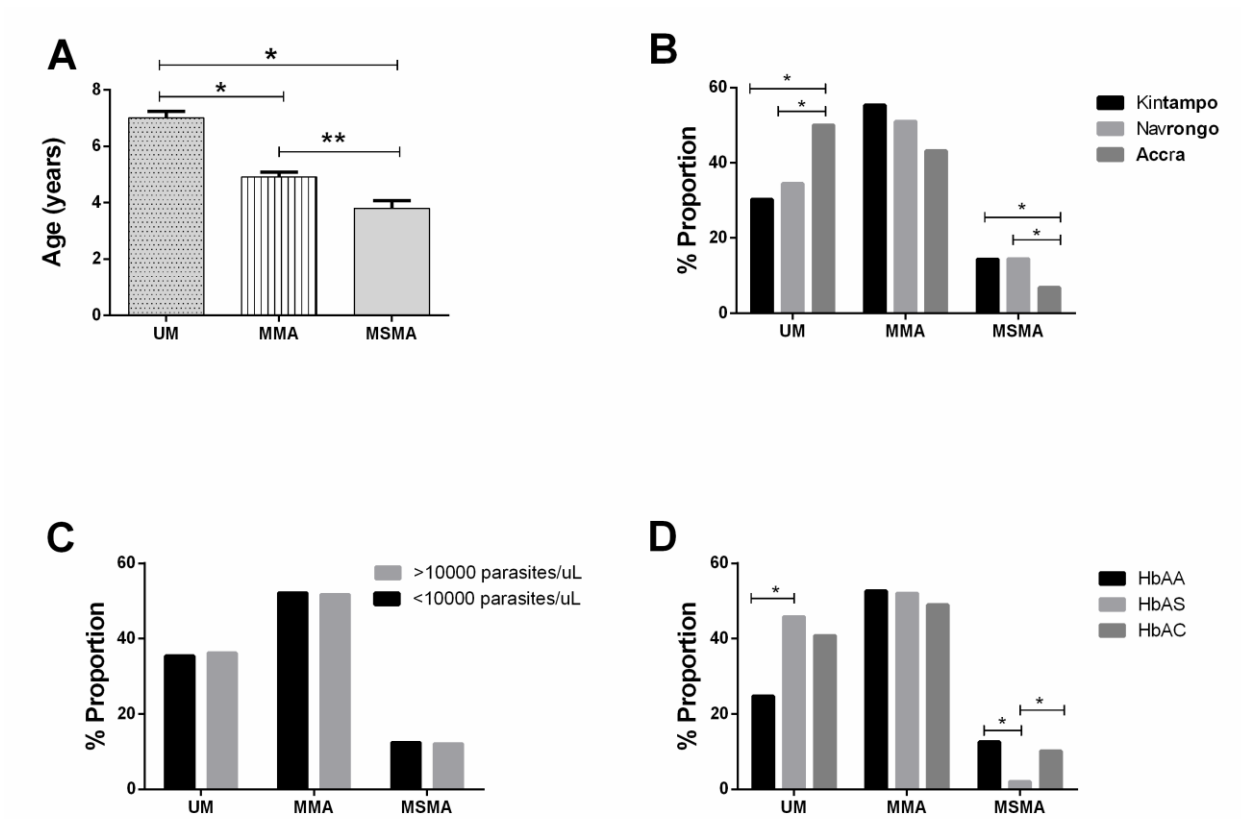
Figure 3.2. Determinants of malarial anaemia severity in children. Children with malaria were divided into three categories based on haemoglobin levels: UM (Hb ≥ 11g/dL, n=194), MMA (8.0 ≤ Hb <11 g/dL, n=279) and MSMA (Hb < 8.0g/dL n=65). The relationships between severity of malarial anaemia and (A) age (B) study area (C) parasite density and (D) haemoglobin genotype were investigated using Kruskal-Wallis test for age and Chi-square analysis for study area, parasite density categories, and haemoglobin genotypes**.**

MCHC levels were significantly lower in children with MMA compared to children with UM (P<0.001); this was not the case in children with MSMA compared to children in the UM group (P=0.075). However, RDW increased with increasing anaemia severity across the groups (P<0.001; Table 3.4), indicting an erythropoietic response. Leucocyte and platelet

indices, including, GRA percent (P<0.001), PLT counts (P<0.001) and PDW (P=0.012) also decreased with increasing anaemia severity, while LYM (P<0.001) and MON (P<0.001) percentages increased across the groups (Table 3.4). WBC counts were not significantly different in any of the malarial anaemia groups when compared to children with UM (Table 3.4).

3.4.6. Socio-demographic predictors of malaria severity

Multiple regression analyses were performed to determine the socio-demographic predictors of haemoglobin level and parasitaemia, as these are the main clinical indicators of malaria severity in this cohort. Although several factors emerged as independent predictors of haemoglobin levels (unadjusted regression coefficients; Table 5), after adjusting for covariates including sickle cell trait, age, sex, father's educational level as well as the occupations of both parents were significant predictors of haemoglobin levels (adjusted regression coefficients; Table 3.5). Females were more likely to have lower Hb levels, whilst children whose fathers had Middle school education or better were less susceptible to anaemia (Table 3.5). Furthermore, our data showed that children whose mothers were teachers, and those whose fathers had any kind of employment, were less likely to be anaemic (Table 3.5). More interestingly, Hb levels were not dependent on site, though children in lower transmission areas had lower parasitaemia.

Significant predictors of parasite density were ecological zone (sampling site) and the use of LLITNs. As expected, children in Navrongo and Kintampo were more likely to have higher parasitaemia, which is consistent with the reported EIR for these areas. Of interest, our analyses showed that higher parasitaemias were associated with the self-reported use of LLITNs, which suggests loss of immunity resulting from reduced exposure to parasites.

Table 3.4. Relationship between haematological indices and severity of anaemia in children with malaria

Haematological indices	UM (N=194) *median*(*IQR*)	MMA (N=279) *median*(*IQR*)	MSMA (N=65) *median*(*IQR*)	Kruskal Wallis *P*-value
Erythrocyte Indices				
RBC (10^3/ mm^3)	4.5 (4.3-4.8)	3.9 (3.5-4.2)	2.5 (2.3-2.8)	<0.001
MCV (µm^3)	77.6 (73.0-81.0)	75.0 (68.7-79.0)	72.0 (65.3-79.8)	0.003
MCH (pg.)	26.2 (24.8-27.4)	24.8 (22.8-26.6)	24.9 (22.1-26.3)	<0.001
MCHC (g/dl)	33.7 (32.5-34.6)	33.1 (32.1-34.3)	33.3 (31.4-34.9)	0.005
RDW (%)	14.8 (13.8-15.7)	15.8 (14.6-17.1)	17.3 (15.4-19.5)	<0.001
Leucocyte indices				
WBC (10^3/mm^3)	8.5 (6.6-11.2)	8.3 (6.1-10.6)	7.6 (4.2-9.8)	0.113
LYM (%)	18.1 (12.0-27.0)	26.7 (18.0-37.2)	39.9 (32.4-48.4)	<0.001
MON (%)	5.7 (3.9-7.7)	6.3 (5.0-8.8)	8.3 (6.6-10.1)	<0.001
GRA (%)	75.6 (65.2-83.6)	66.6 (54.7-76.0)	51.0 (45.1-60.1)	<0.001
Platelet Indices				
PLT (10^3/mm^3)	168.0 (112.3-229.0)	119.0 (78.0-180.0)	117.0 (69.3-177.8)	<0.001
MPV (µm^3)	7.9 (7.2-9.0)	7.6 (7.0-8.3)	7.7 (7.2-8.3)	0.005
PDW (%)	14.7 (12.6-17.0)	14.4 (11.6-16.6)	13.5 (10.1-15.5)	0.012

Data are presented as medians (first quartile-third quartile).

Table 3.5. Simple and multiple linear regression analysis of factors associated with haemoglobin and log parasitaemia in malaria positive children

	Haemoglobin level in g/dL				Log Parasite density			
	β^* (95%CI β^*)	P-value	β^{**}	P-value	β^* (95%CI β^*)	P-value	β^{**} (95%CI β^{**})	P-value
Age	0.21 (0.16, 0.25)	<0.0001	0.19 (0.14, 0.24)	<0.001	-0.07 (-0.09, -0.03)	<0.0001	-0.07 (-0.10, -0.05)	0.002
Sex								
Male	ref		ref		ref		ref	
Female	-0.29 (-0.61,0.03)	0.079	-0.30 (-0.61, -0.01)	0.055	-0.09 (-0.25, 0.07)	0.267	-0.01 (-0.17, 0.15)	0.896
Ecological Zones								
Accra	ref		ref		ref		ref	
Navrongo	-0.69 (-1.11, -0.34)	<0.001	0.10 (-0.54, 0.73)	0.769	0.29 (0.11, 0.51)	0.003	0.30 (-0.03, 0.63)	0.070
Kintampo	-0.90 (-1.26, -0.52)	<0.001	-0.27 (-0.92, 0.38)	0.418	0.31 (0.11, 0.46)	0.002	0.41(0.08, 0.75)	0.014
Mother's Education								
None	ref		ref		ref		ref	
Primary	-0.13 (-0.36, 0.62)	0.603	-0.49(-1.00, 0.03)	0.064	-0.28 (-0.50, -0.02)	<0.050	-0.10 (-0.34, 0.20)	0.447
Middle	0.30 (-0.43, 1.03)	0.425	-0.60 (-1.31, 0.12)	0.104	-0.11 (-0.47, 0.26)	0.563	0.05 (-0.32, 0.43)	0.776
JHS	0.39 (-0.02, 0.79)	0.062	-0.32 (-0.79, 0.15)	0.184	-0.10 (-0.29, 0.10)	0.348	0.03 (-0.20, 0.29)	0.799
Vocation	0.80 (-0.33, 1.94)	0.165	-0.56 (-1.70, 0.59)	0.339	-0.23 (-0.81, 0.35)	0.432	0.16 (-0.43, 0.75)	0.603
Secondary	0.70 (-0.15, 1.24)	0.012	-0.40 (-1.05, 0.25)	0.223	-0.08 (-0.35, 0.19)	0.571	0.01 (-0.32, 0.35)	0.999
Tertiary	1.00 (-0.13, 2.14)	0.083	-0.57 (-1.95, 0.81)	0.416	-0.06 (-0.62, 0.50)	0.830	-0.22 (-0.91, 0.51)	0.551
Father's Education								
None	ref		ref		ref		ref	
Primary	0.24 (-0.32, 0.79)	0.400	0.27 (-0.27, 0.81)	0.326	0.02 (-0.26, 0.29)	0.991	0.04 (-0.24, 0.32)	0.790
Middle	1.01 (0.51, 1.64)	<0.0001	0.77 (0.18, 1.36)	0.010	0.12 (-0.19, 0.43)	0.666	0.12 (-0.19, 0.43)	0.436
JHS	0.64 (0.17, 1.12)	<0.0001	0.35 (-0.20, 0.91)	0.212	-0.10 (-0.39, 0.19)	0.034	-0.11 (-0.39, 0.17)	0.442
Vocation	0.21 (-1.27, 1.69)	0.780	0.74(-0.67, 2.16)	0.304	-0.79(-1.52, 0.06)	0.299	-0.78 (-1.51, -0.05)	0.036
Secondary	1.05 (0.60, 1.51)	<0.0001	0.64 (0.10, 1.18)	0.021	0.03 (-0.26, 0.31)	0.397	0.04 (-0.24, 0.32)	0.770
Tertiary	1.12 (0.56, 1.68)	<0.0001	0.43 (-0.41, 1.27)	0.318	0.17 (-0.26, 0.61)	0.456	0.18 (-0.25, 0.62)	0.406
Mother's Occupation								
Unemployed	ref		ref		ref		ref	
Farmer	-0.60 (-1.17, -0.03)	0.041	-0.18 (-0.79, 0.43)	0.558	0.26 (-0.02, 0.55)	0.070	0.14 (-0.18, 0.45)	0.390
Teacher	0.82 (0.20, 1.41)	0.114	1.27 (0.23, 2.31)	0.017	0.38 (-0.12, 0.89)	0.138	0.49 (-0.05, 1.03)	0.074
Trader	0.47 (-0.07, 1.0)	0.088	0.41 (-0.12, 0.94)	0.127	0.12 (-0.14, 0,39)	0.368	0.17 (-0.10, 0.45)	0.208
Other	0.14 (-0.47, 0.75)	0.654	0.18 (-0.45, 0.81)	0.567	0.17 (-0.14, 0.48)	0.276	0.18 (-0.11, 0.53)	0.204
Father's Occupation								
Unemployed	ref				ref		ref	
Farmer	0.28 (-0.815, 1.38)	0.613	1.32 (0.08, 2.56)	0.037	0.15 (-0.41, 0.71)	0.602	0.08 (-0.56, 0.72)	0.807
Teacher	1.41 (0.22, 2.60)	0.020	1.84 (0.48, 3.20)	0.008	-0.01 (-0.61, 0.63)	0.983	-0.08 (-0.79, 0.62)	0.818
Trader	0.67 (-0.46, 1.80)	0.247	1.33 (0.09, 2.57)	0.036	-0.01 (-0.59, 0.58)	0.990	0.01 (-0.64, 0.65)	0.909
Other	1.00 (-0.11, 2.10)	0.077	1.24 (0.01, 2.46)	0.048	0.02 (-0.55, 0.58)	0.959	0.13 (-0.51, 0.76)	0.456
Professional	0.91 (-0.31, 2.14)	0.143	1.24 (-0.16, 2.65)	0.082	-0.06 (-0.70, 0.58)	0.863	-0.07 (-0.79, 0.66)	0.955

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TABLE 5 continued

	Haemoglobin level in g/dL				Log parasite density			
	$\beta^*(95\%CI\ \beta^*)$	P-value	$\beta^{**}(95\%CI\ \beta^{**})$	P-value	$\beta^*(95\%CI\ \beta^*)$	P-value	$\beta^{**}(95\%CI\ \beta^{**})$	P-value
Cement block house								
Yes	ref		ref		ref			
No	-0.40 (-0.72, -0.0)	0.015	-0.07 (-0.44, 0.30)	0.707	-0.11 (-0.05, 0.26)	0.190	0.20 (-0.01, 0.39)	0.045
Thatched house								
Yes	ref		ref		ref			
No	0.64 (0.33, 0.96)	<0.0001	0.17 (-0.24, 0.57)	0.420	-0.03 (-0.19, 0.12)	0.696	0.10(-0.11, 0.31)	0.356
Mosquito control measures								
Bed net use	ref		ref		ref		ref	
No Bed net use	0.69 (0.36, 1.02)	<0.0001	0.30 (-0.09, 0.69)	0.131	-0.40 (-0.56, -0.24)	<0.001	-0.32 (-0.52, -0.12)	0.002
Own farm								
Yes	ref		ref		ref		ref	
No	0.83 (0.502, 1.16)	<0.0001	0.03 (-0.44, 0.49)	0.917	-0.14 (-0.30, 0.03)	0.113	0.21 (-0.03, 0.44)	0.092
Clinical factors								
Parasite Density (parasites/μL)	$-1 \times 10^{-6}(-2 \times 10^{-6}, -1 \times 10^{-7})$	0.003	$1 \times 10^{-7}(-1.6 \times 10^{-6}, 1 \times 10^{-7})$	0.593	N /A	N/A		
Haemoglobin type								
AA	ref		ref		ref		ref	
AS	0.48 (-0.06, 1.02)	0.084	0.61 (0.053, 1.16)	0.032	-0.157 (-0.43, 0.11)	0.254	-0.20 (-0.48, 0.09)	0.177
AC	-0.01 (-0.55, 0.53)	0.972	0.06 (-0.46, 0.59)	0.813	0.192 (-0.08, 0.46)	0.158	0.24 (-0.03, 0.51)	0.085

N/A is Not Applicable.

ref represent the reference category.

$\beta^*(95\%CI\ \beta^*)$ and $\beta^{**}(95\%CI\ \beta^{**})$ represent unadjusted and adjusted regression coefficient and their confidence intervals respectively.

3.5. Discussion

As a result of concerted intervention strategies, malaria transmission in endemic areas has been declining in the last decade, resulting in a cumulative drop in the incidence of malaria cases worldwide (World Health Organization., 2016). However, since clinical manifestations of malaria appear to be significantly influenced by transmission intensity (Greenwood, 1997, Snow *et al.*, 1997), the impact of the decreasing transmission on clinical and haematological indices during malaria should be an important consideration in planning for the long-term management of the disease. Using the varying transmission intensity across three well-characterized areas in Ghana as a model, we have demonstrated that decreasing transmission leads to a shift in the vulnerable group to slightly older children. This shift was accompanied by a reduced disease severity in children with malaria, characterized by lower parasitaemias, higher haemoglobin levels and higher erythrocyte counts. Previous studies conducted in high transmission areas have shown that anaemia severity was not associated with parasite burden (Ong'echa *et al.*, 2006, Novelli *et al.*, 2010). Our study confirms that high density parasitaemia is not necessarily a predictor of anaemia severity, as the proportion of children with and without high density parasitaemia was not different in each of the three categories of malaria. However, studies in low transmission settings have shown a correlation between parasite density and malaria severity (Phillips *et al.*, 2009, Tangpukdee *et al.*, 2012), indicating the importance of transmission intensity as an important factor in determining malaria severity. Although parasite density did not significantly correlate with haemoglobin levels in any of the three sites in this study, the relationship between the two was still evident in the data showing that children in the higher transmission areas (Kintampo and Navrongo) had much higher parasitaemias and were more susceptible to moderate-to-

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severe anaemia compared to those in Accra. This may suggest a complex interplay of factors involving transmission intensity, parasite densitiy, and haemoglobin levels.

Malarial anaemia severity was significantly associated with erythropaenia, lymphocytosis, monocytosis, agranulosis, reticulocytosis and thrombocytopenia. Thrombocytopenia is commonly associated with malaria, and is a marker of malaria severity (Erhart *et al.*, 2004, Mahmood and Yasir, 2008, Novelli *et al.*, 2010). Reduction in platelet count has been associated with splenomegaly (Novelli *et al.*, 2010), a complication in severe malaria that may lead to death. Consistent with previous reports (Allen *et al.*, 1992, Kreuels *et al.*, 2010, Lell *et al.*, 1999, Stirnadel *et al.*, 1999, Williams *et al.*, 2005b), we found evidence of the protective effect of sickle cell trait against malarial anaemia. Children with the HbAS genotype had a significantly lower rate of MSMA and higher rate of UM compared to those with normal haemoglobin genotype (HbAA). These patterns were not observed in the HbAC genotypic group, indicating that the mechanisms of protection of the two haemoglobinopathies are likely distinct. *In vitro* studies have demonstrated that there is inhibition of parasite growth and reduced invasion into HbS erythrocytes (Friedman, 1978, Pasvol *et al.*, 1978), and that sickle cell trait accelerates the acquisition of natural immunity (Williams *et al.*, 2005a). Additional analyses of parasitaemia levels showed that the protective effect of HbAS on parasite density appeared to be specific to only the holoendemic area (Kintampo). This apparent disappearance of the protective effect in lower transmission areas may be a potentially important finding, with significant implications for susceptibility to severe disease in sickle cell carriers in endemic areas as control interventions continue to reduce transmission.

Multivariate analysis revealed that the educational status of the father as well as the economic status of the household are major predictors of the severity of malarial anaemia.

Typically, in African households, decision-making is the responsibility of the father and hence his level of education and understanding of malaria, as well as the availability of funds would determine whether healthcare would be sought early. Recent reports by Diiro and colleagues in Kenya have shown that households headed by males with a higher level of education were at lower risk of malaria (Diiro *et al.*, 2016). Children of parents with less than secondary level education are more likely to come from low economic backgrounds, which has been shown to put these children at higher risk of severe malaria (Houngbedji *et al.*, 2015).

Protection against malaria is acquired by continuous exposure to the parasites (Doolan *et al.*, 2009). Studies in northern Ghana has shown that the efficacy of naturally acquired immunity in protecting against high density parasitaemia infections was 94% (Owusu-Agyei *et al.*, 2001). Bed net use is associated with reduced incidence of malaria morbidity and mortality (Afoakwah *et al.*, 2015, Alonso *et al.*, 1991, Binka *et al.*, 1996, Lindblade *et al.*, 2015, Müller *et al.*, 2006, Phillips-Howard *et al.*, 2003). Snow and colleagues suggested that whilst interventions such as LLITNs may diminish the incidence of malaria infection, they may in turn increase the incidence of high density parasitaemia infections and poor clinical outcomes (Snow *et al.*, 1997). Our data supports this argument, showing that children who used LLITNs were at a higher risk of having high density parasitaemia possibly due to a lack of contact with the parasite, leading to little naturally acquired immunity.

3.6. Conclusion

The results presented in this study demonstrate significant changes in the clinical manifestations as well as the determinants of diseases severity of malaria as transmission intensity decreases. Children living in households where the head-of household had Middle school education or better were less likely to develop high density parasitaemia. The protective effect of sickle cell trait against severe malarial anaemia becomes less profound with reduction in transmission intensity. Additionally, our data suggests children who used LLITNs were at a higher risk of developing high density parasitaemia, possibly due to delayed acquisition of immunity, which may lead to a rise in the incidence of fatally severe cases of malaria.

CHAPTER FOUR

4.0 Paper 2: Antibody reactivity to *Plasmodium falciparum* invasion ligands in children with malaria

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4.1. Abstract

Plasmodium falciparum uses a repertoire of merozoite-stage proteins for invasion of erythrocytes. Antibodies against some of these proteins halt the replication cycle of the parasite by preventing erythrocyte invasion and are implicated as contributing to acquired immune protection against malaria. Natural acquisition of antibodies against merozoite antigens is influenced by diverse factors, which complicate the evaluation of antigen candidacy for vaccine development. Here, we examine antibody reactivities against key invasion proteins in children with malaria across three areas of varying endemicity in Ghana. Plasma samples from 421 children (aged 2-14 years) with malaria in three areas of Ghana with different levels of malaria endemicity were tested by ELISAs for antibodies to *P. falciparum* invasion antigens, including erythrocyte binding antigens (EBA) 175, EBA140, EBA181, and reticulocyte binding-like homologue (Rh) 2, Rh4 and Rh5. In addition, antibodies against invasion antigens were negatively correlated with parasitaemia, and increased in an age-dependent manner. Regression analysis revealed that the strongest predictors of breadth of antibody reactivity were exposure and age. Altogether, the data suggest that patterns of antibody responses against invasion proteins are both antigen and exposure dependent. Deeper understanding of how these factors interplay may be important in the identification of potential blood stage vaccine targets.

4.2. Introduction

The development of an effective vaccine that can elicit production of broad, strain-transcending neutralizing antibodies may be the key to the elimination of malaria. The most advanced malaria vaccine candidate, RTS,S elicits high levels of antibodies to the circumsporozoite protein that are correlated with partial protection that operates at the pre-erythrocytic stage, but this wanes over several months post vaccination (Olotu *et al.*, 2013, Olotu *et al.*, 2016). Nonetheless, acquisition of immunity by adults living in endemic areas as well as the ability of immune sera from malaria-exposed adults to clear parasitemia in malaria-infected children (Cohen *et al.*, 1961) gives a strong indication that immunity against malaria is possible. Unfortunately, limited understanding of mechanisms of protective immunity to malaria constrains efforts towards vaccine development.

The asexual blood stage of infection is responsible for the clinical manifestation of malaria, and a vaccine against parasites at this stage may reduce malaria severity even if they do not prevent infection entirely. Erythrocyte binding antigen (EBA) and reticulocyte-like binding homologue (Rh) proteins are involved in erythrocyte invasion (Cowman and Crabb, 2006), a crucial step in the asexual blood stage of the parasite. EBA and Rh proteins are deployed hierarchically during invasion and show significant variation in expression levels in clinical isolates (Bowyer *et al.*, 2015, Mensah-Brown *et al.*, 2015), both of which are thought to be mechanisms of evading the host immune system. Antibodies against EBA and Rh proteins, can inhibit parasite growth *in vitro* (Reiling *et al.*, 2012, Chiu *et al.*, 2014, Chiu *et al.*, 2015, Triglia *et al.*, 2011, Tran *et al.*, 2014), inhibit erythrocyte binding by merozoites (Irani *et al.*, 2015), fix complement (Boyle *et al.*, 2015) and opsonize merozoites (Osier *et al.*, 2014a, Abdalla, 1989, Hill *et al.*, 2013, Joos *et al.*, 2010), emphasizing the key role of these antigens in the acquisition of humoral immunity and protection against malaria.

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Humoral immunity to malaria is influenced by diverse factors including age and local transmission intensity which both influence individual cumulative exposure to infection (Akpogheneta *et al.*, 2008, Noland *et al.*, 2015, Stanisic *et al.*, 2015), complicating the identification of specific targets of immunity for vaccine development. Recent reports indicate that humoral immune responses are more likely to be protective when targeted against multiple antigens (Abdalla, 1989, Dent *et al.*, 2015, Osier *et al.*, 2014b, Richards *et al.*, 2013). While acquisition and breadth of antibody reactivity affect clinical outcome of disease (Abkarian *et al.*, 2011, John *et al.*, 2005, Osier *et al.*, 2008, Richards *et al.*, 2010), the determinants of these in endemic areas remain unclear.

In this study, the relationships between humoral immune responses to blood stage malaria and transmission intensity, age and parasitemia were investigated by examining antibodies against a panel of merozoite stage invasion-related antigens in children with malaria residing in three areas of differing levels of endemicity in Ghana.

4.3. Study participants and methods

4.3.1. Study sites and sampling

Plasma was obtained from blood samples of children aged between 2-14 years with clinical malaria in three ecologically distinct areas in Ghana, namely: Ledzokuku Krowor municipality (LEKMA) in Accra; the Kassena and Nankana districts in Northern Ghana, and the Kintampo Municipality in the middle belt of Ghana, as described in detail elsewhere (Mensah-Brown *et al.*, 2017, Mensah-Brown *et al.*, 2015). The local malaria transmission intensity as previously measured by the entomological inoculation rates (EIR) is highest in Kintampo, followed by Navrongo, and lowest in Accra (Kintampo>Navrongo>Accra) (Dery *et al.*, 2010, Kasasa *et al.*, 2013, Klinkenberg *et al.*, 2008).

4.3.2. Sample size considerations

Sample size projections were based what will be required to adequately examine the patterns of antibodies to merozoite antigens across areas with different transmission inensity. A sample size of 124 children was required to detect correlations with coefficient ≥0.25 at >80% power and α=0.05 level. Therefore, samples were collected from at least 124 children at each site for a minimum of 372 across all sites, which allowed analyses to be performed at two levels: at the zonal level and a meta-analysis encompassing all the zones to obtain a comprehensive country-wide profile.

4.3.3. Recombinant antigens

A recombinant antigen panel based on sequences of key merozoite invasion ligands was used for assays to study antibodies in plasma samples. Five of the antigens, Regions III-V of EBA140 (3D7; amino acids 770-1064), regions III-IV of EBA175 (3D7; amino-acids, 761-1298), a portion of the EBA181 sequence (3D7; amino-acids 769-1365), Rh2A9 (3D7;

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amino acids 2027 to 2533, common region for both Rh2a and Rh2b) and Rh4.2 (amino acids 1277–1451), were expressed in *Escherichia coli* cells and purified using glutathione-agarose beads (Reiling *et al.*, 2010, Reiling *et al.*, 2012, Richards *et al.*, 2010). Full-length ectodomains of EBA140, EBA175, EBA181 and Rh5, were expressed as biotinylated proteins in human embryonic kidney (HEK) 293E cells and purified by nickel affinity chromatography (Crosnier *et al.*, 2013).

4.3.4. Quantitation of antigen-specific antibodies in plasma

Enzyme linked immunosorbent assays (ELISAs) were used to determine the relative levels of antigen-specific antibodies in plasma samples. Levels of IgG antibodies to the GST-tagged antigens (EBA140 RIII-V, EBA175 RIII-V, EBA181 RIII-V, RH2-2030, and Rh4.2) were measured as previously described [37], with slight modifications. Briefly, wells of flat bottom 96-well plates (Immulon 4HBX, Thermo Scientific) were coated at 0.50 µg/mL concentration of individual recombinant antigens (GST-tagged antigens) in 50 µL of coating buffer (15 mM Na2CO3, 35 mM NaHCO3, pH 9·3) and incubated overnight at 4°C. The plates were washed with phosphate buffered saline (PBS) containing 0·05% v/v Tween 20 (PBS-T) and blocked for 3 hours with 1% skimmed milk (Marvel™ UK) in PBS-T at room temperature. Plates were washed prior to addition of 50 µL of plasma, diluted 1/500 in blocking buffer. After incubation at 4°C overnight, the plates were washed and then incubated with 100 µL of horseradish peroxidase-conjugated rabbit anti-human IgG (at a dilution of 1/15,000) (Dako Ltd.), washed again, before 100 µL of tetramethylbenzidine substrate (TMB) solution was added. After incubation for 20 minutes, reactions were stopped using 50 µL of 0.2 M sulphuric acid, and optical densities were measured at 450 nm

in a microplate reader (Bio-Rad iMark, Hertfordshire, UK). All plasma samples were assayed in duplicates and blank wells were set up using GST only.

For the biotinylated antigens, the ELISA procedure was as previously described (Osier *et al.*, 2014b), with minor modifications. Streptavidin-coated 96-well ELISA plates (NUNC, Denmark) were washed with PBS-T and blocked for 30 minutes with PBS containing 0.5% bovine serum albumin (BSA) at room temperature, prior to coating with individual recombinant antigens at 0.50 µg/mL concentration in X volume. Antigen-coated plates were incubated at room temperature for 45 minutes and washed with PBS-T. Plasma samples were added at 1:1000 dilution in blocking buffer and incubated overnight at 4°C. The plates were washed and incubated with 100 µL of horseradish peroxidase-conjugated rabbit anti-human IgG (at a dilution of 1/15,000) (Dako Ltd.) prior to addition of TMB substrate. Reactions were stopped and optical densities were measured as described above. Antibody reactivities of 20 nonimmune individuals (malaria non-exposed Europeans) were tested as negative controls in all assays. Antibody reactivity of a sample was considered positive if the OD was greater than mean +3 standard deviations of these control individuals.

4.3.5. Statistical Analysis

Data were analysed using Minitab (version 17). Kolmogorov-Smirnov tests were used to determine normality of the data before application of further tests. One-way ANOVA or Kruskal-Wallis tests were applied for across group comparisons for normally and non-normally distributed data respectively. Post-hoc tests of either Student t-test or Mann-Whitney U tests were applied for pairwise analysis where necessary. For all analyses, $P<0.05$ was considered statistically significant.

4.4. Results

4.4.1. Demographic data and parasitaemia levels of children with malaria

Plasma IgG antibodies to a panel of *P. falciparum* invasion-related antigens were measured in 421 children with malaria from three different communities across Ghana. As previously described in other analyses on these individuals (Mensah-Brown *et al.*, 2017), malaria positive children in Accra (N=125) were significantly older than those recruited in Navrongo (N=131; *P*=0.002, Table 4.1). No significant differences were observed in the ages of children recruited in Kintampo compared to children living in both Accra and Navrongo. Comparison of sex distribution across the three sites showed that there was no statistically significant difference across the three sites (*P*=0.419; Table 4.1). Parasite densities differed significantly across the three sites (*P*<0.001), with children from Kintampo showing the highest parasitaemias, followed by Navrongo and then Accra, mirroring the transmission intensities in these areas.

Table 4.1. Clinical and demographic characteristics of malaria-positive Ghanaian children aged 2-14 years in Kintampo, Navrongo and Accra at the time of sampling

	Kintampo	Navrongo	Accra	Total	P Value
No. of enrolees	167	131	125	421	
Female (%)	46.71	52.10	43.52	47.46	0.419[a]
Age, years	5.7 (0.3)	5.1 (0.3)	6.4 (0.3)	5.7 (0.2)	0.009[b]
Parasitaemia, parasites/µL	137, 824 (17, 297)	46, 863 (4, 533)	41,184 (3, 876)	82,189 (7,601)	<0.001[b]

Relative malaria transmission intensities ficantas measured by entomological inoculation rates in the areas are in the order Kintampo>Navrongo>Accra

Data for age and parasitaemia are presented as means (standard error of the mean, SEM).

[a]P-value obtained from Chi Square Test, [b]P-value obtained from ANOVA. $P \leq 0.05$ was considered statistically significant.

4.4.2. Antibody levels of children with malaria to invasion antigens

To understand whether there were differences in antibody levels of children with malaria living in areas of differing transmission intensity, ELISAs antibody levels to all antigens tested were compared across the three sites. Whilst no significant differences were observed in antibody levels to EBA175, EBA140, EBA 181 RIII-V and Rh5, significant differences were observed in EBA181 FL, Rh2.2030 and Rh4.2 antibodies. Antibody levels to EBA181 FL were higher in children from Kintampo and Navrongo compared to children from Accra ($P<0.05$ for both comparison; Figure 4.1). Similarly, antibody levels to Rh2.2030 were significantly higher in children living in Kintampo compared to children living in Accra ($P=0.006$), though no difference was observed in comparison to children from Navrongo

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(P=0.234). Conversely, children in Accra showed higher antibody levels to Rh4.2 compared to children living in Navrongo and Kintampo (P<0.05 for both comparison; Figure 1).

4.4.3. Age-dependent acquisition of antibodies against EBA and Rh proteins

In endemic areas, age is often a surrogate marker for exposure; therefore, we categorized malaria-positive children into two age categories, ages 2-5 and 6-14 years old. We observed an age-associated increase in IgG to RIII-V of EBA140, EBA175, EBA181, and full ectodomains of EBA175 and Rh5 (P<0.05 for all comparisons; Figure 4.2). Antibody levels to full ectodomains of EBA175, EBA181 and Rh4.2 also increased in an age-dependent manner (P<0.05 for all comparisons; Figure 4.2). We however did not observe an age associated trend in Rh2.2030 (P=0.174) (Figure 4.2).

We further determined whether antibody levels were associated with age within the different populations. In Kintampo, antibody levels to EBA175 RIII-V and full length EBA140 were significantly higher in children between 2 and 5 years of age compared with children who were older (P=0.001 and P=0.012 for EBA175 RIII-V and full length EBA140 respectively; Figure 4.3A) While age-associated trend was observed for the other antigens tested, these were not significant (P=0.103, P=0.186, P=0.663, P=0.254, P=0.466, P=0.161, P=0.086 for EBA140 RIII-V, EBA181 RIII-V, EBA175 FL, EBA181 FL, Rh2, Rh4 and Rh5 FL respectively; Figure 4.3A). For children resident in Navrongo, a significant age-dependent trend was observed in antibody levels to EBA181 FL (P=0.027; Figure 4.3) and Rh2(P=0.040; Figure 4.3B). Antibody levels between age groups for the other antigens tested were not significantly different among children in Navrongo (P=0.278, P=0.903, P=0.596, P=0.406, P=0.590, P=0.392, P=0.288 for EBA140 RIII-V, EBA175 RIII-V, EBA181 RIII-V, EBA140 FL, EBA175 FL, Rh4 and Rh5 FL respectively; Figure 4.3B).
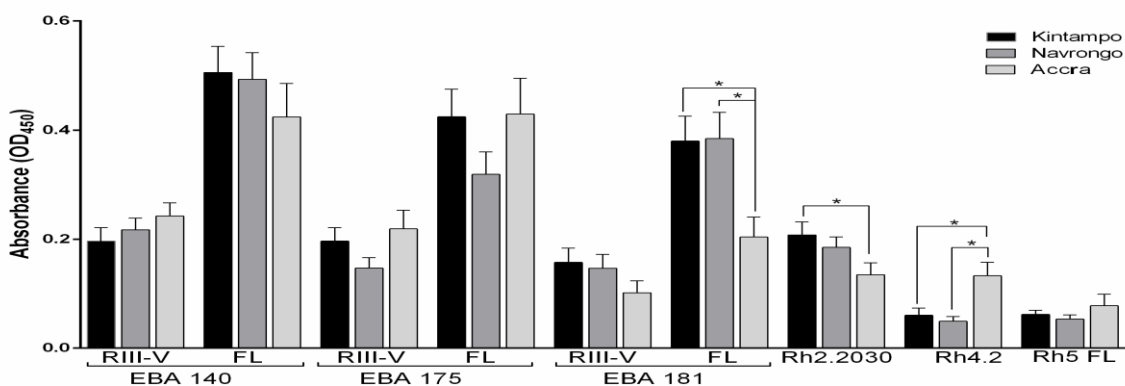
Figure 4.1 Mean antibody levels of children with malaria across three endemic regions of Ghana. Plasma IgG levels measured by ELISA ODs to EBA and Rh proteins of malaria-positive children resident in Kintampo (N=167), Navrongo (N=131), and Accra (N=128). Bars represent mean antibody levels within groups. the Error bars represent SEMs. * means (P< 0.05) using Mann-Whitney U test for comparison. FL=full length.
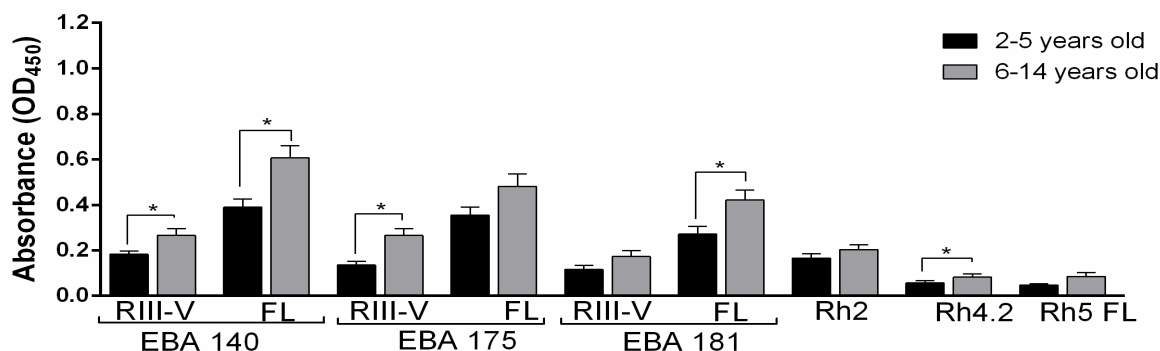
Figure 4.2. Age-dependent acquisition of IgG against recombinant EBA and Rh proteins. ELISAs were conducted using plasma from malaria positive children (N=423). living in Ghana. IgG levels to recombinant EBA and Rh antigens were grouped into two age group: 2-5 years old and 6-14 years old. Bars represent group means. Error bars represent SEM. * means (P< 0.05) using student t test for comparison. FL=full length.
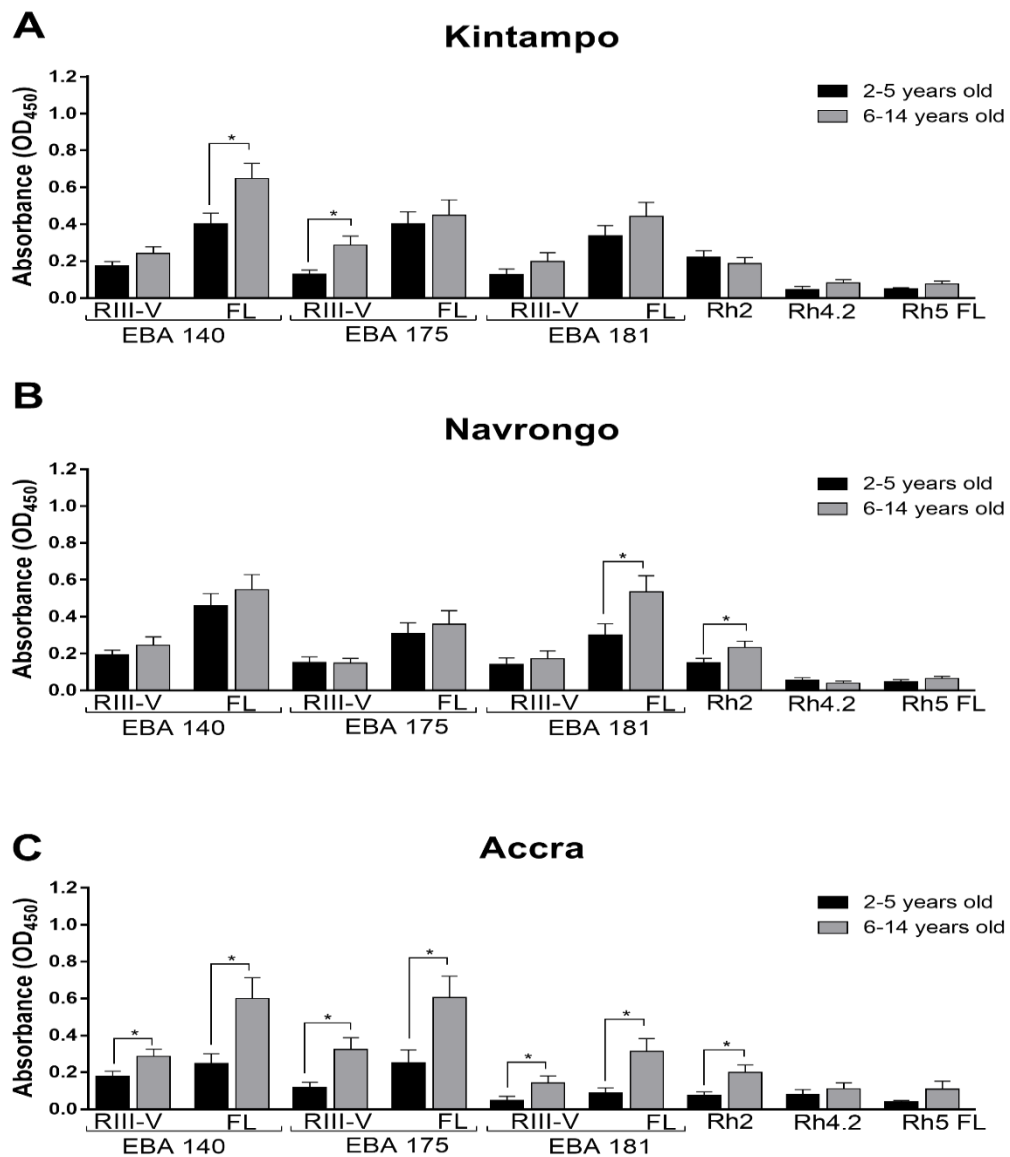
Figure 4.3. Age-dependent acquisition of IgG against recombinant EBA and Rh proteins across three endemic areas. ELISAs were conducted using plasma from malaria positive children living in (A) Kintampo (*N*=167), (B) Navrongo (*N*=131), and (C) Accra (*N*=128. IgG levels to recombinant EBA and Rh antigens were grouped into two age group: 2-5 years old and 6-14 years old. Bars represent group means. Error bars represent SEM. * means (P< 0.05) using student t test for comparison. FL=full length.

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Antibody levels to RIII-V and full length of the EBAs and Rh2 were significantly lower in younger children compared to older children in Accra (P=0.040, P=0.107, P=0.009, P=0.017, P=0.050, P=0.006, P=0.012 for EBA140 RIII-V, EBA175 RIII-V, EBA181 RIII-V, EBA140 FL, EBA175 FL, EBA181 FL, and Rh2 respectively; Figure 4.3C). We did not observe a significant higher in levels of Rh4 (P=0.469; Figure 4.3C) and Rh5 FL (P=0.162; Figure 4.3C) in older children compared younger children living in Accra

4.4.4. Seropositivity to invasion antigens across three endemic sites

Seropositivity to all nine antigens tested was compared for the children recruited across the three endemic sites. We observed no significant difference in the seroprevalence of antibodies to RIII-V of EBA140, EBA175, EBA181 and full length Rh5 (Chi-square; P=0.735, P=0.212, P=0.068 and P=0.175 respectively; Figure 4.4).

A significant lower proportion of children in Accra (~43%) were seropositive for antibodies to EBA140 FL compared to children living in Kintampo (P=0.016; Chi-square) and Navrongo (P=0.004; Chi-square), though no difference was observed in proportion of seropositive children living in Kintampo compared to Navrongo (P=0.532; Chi-square).

A similar trend was observed with regards to the seroprevalence of antibodies to EBA181 FL and Rh2, where significant differences were observed in the proportion of seropositive children in Accra in comparison to children in Kintampo (P<0.001 and P=0.008 for EBA181 FL and Rh2 respectively; Chi-square), and Navrongo (P<0.001, and P=0.001 for EBA181 FL and Rh2 respectively; Chi-square), but not Kintampo compared to Navrongo. Proportion of children who tested positive for antibodies to EBA175 FL was higher in Navrongo compared to Accra (P=0.024; Chi-square), with no difference observed between Accra and Kintampo (P=0.090; Chi-square), or Kintampo and Navrongo (P=0.485; Chi-square).

The proportion of children seropositive to Rh4 was significantly higher in Accra compared to Kintampo (P=0.008; Chi-square). The proportion of children seropositive to Rh4 was not different in children living Navrongo compared to Kintampo (P=0.269; Chi-square) and Accra (P=0.144; Chi-square).

4.4.5. Relationship between breadth of antibody reactivity, age, parasite density and exposure

Multiple studies have suggested that breadth of antibody reactivity is not only a better correlate of protection but also an important predictor of disease outcome (Osier *et al.*, 2014b, Osier *et al.*, 2008). Breadth was estimated as the number of antigens recognized for each child. The proportion and distribution of breadth of antibody reactivity was compared across the three endemic areas. Accra was severely skewed to the left (skewness = 0.75, kurtosis= -0.65; Figure 4.5), with majority of children recognizing 0-2 of the antigens. In contrast, the distribution in Kintampo was only slightly skewed to the left (skewness= 0.60, kurtosis= -0.16), with a broader range of antigen recognition, ranging from 1-5 antigens. The distribution in Navrongo showed high kurtosis (skewness= 0.19, kurtosis= -0.99, Figure 4.5), with no clear peak and low frequencies of recognition for 0-6 antigens. Breadth of antibody reactivity was positively correlated with age (R=0.225, P<0.001) and negatively correlated with parasite density (ρ=-0.115, P=0.002). Regression analysis showed that the strongest predictors of breadth of antibody reactivity were age (F= 24.01, P<0.001) and transmission area (F= 4.80, P=0.009).

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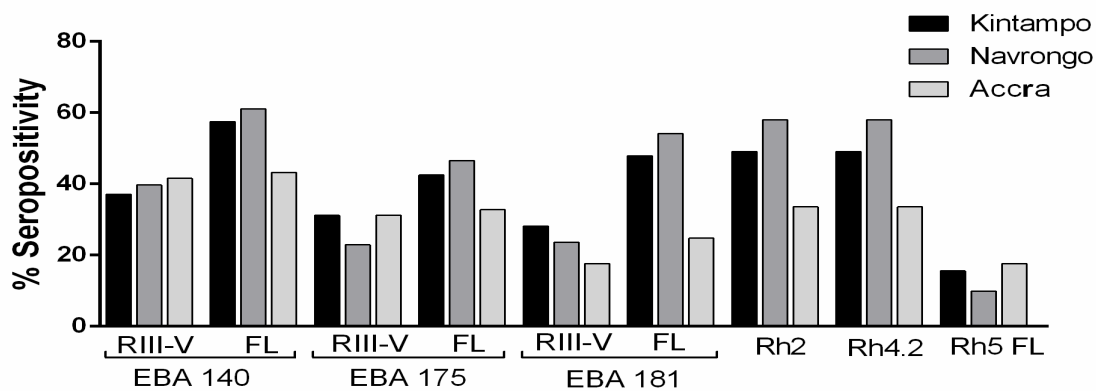
Figure 4.4. Seroprevalence of antibodies against different EBA and Rh proteins in children with malaria across three areas with different transmission intensities. The proportion of children living in Kintampo (N=167), Navrongo (N=131) and Accra (N=128) that were seropositive to EBA and Rh antigens was determined by ELISA. Antibody reactivity of a sample was considered positive if the OD was greater than mean +3 standard deviations of these control individuals. FL=full length.

4.4.6. Relationship between relative antibody levels and transmission intensities.

In order to understand the dynamics of how transmission intensity affects the antibody levels to EBA and Rh proteins, we expressed the response to each antigen as a proportion of the total response for each child in order to compare the patterns of antigen-specific antibodies across three transmission areas. Relative antibody levels to full length EBA 140, 175 and 181, as well as EBA175 RIII-V and Rh2.2030 increased in an exposure-dependent manner when compared across the three transmission areas, though only full length EBA 181 and Rh2 showed significant differences between the three endemic areas. Relative proportion of antibodies to full length EBA181 increased with exposure, as children living in Kintampo showed significantly higher proportions of these antibodies compared to children living in

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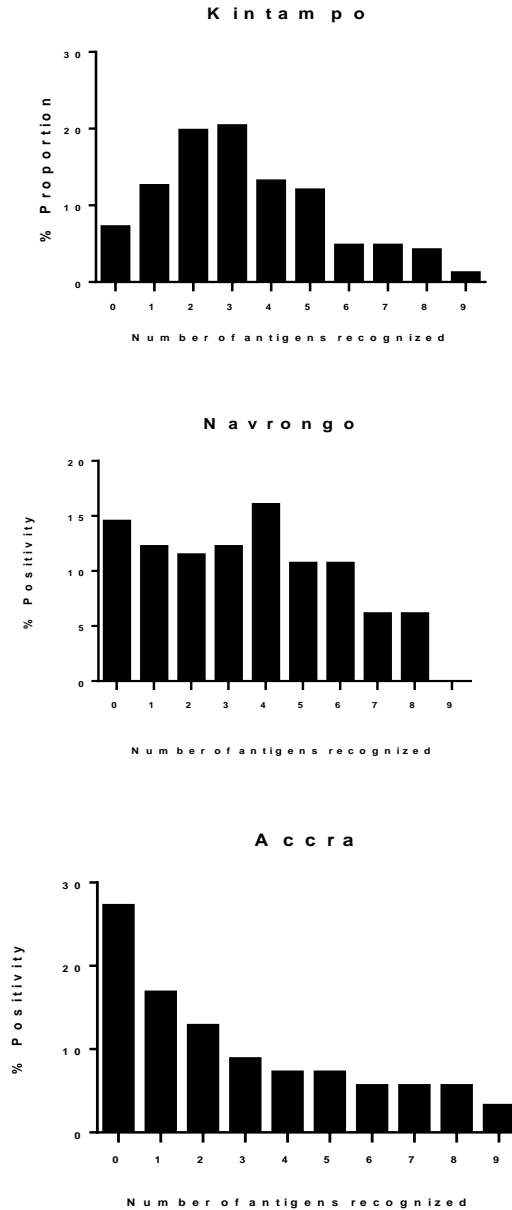
Figure 4.5. The breadth of response to multiple antigens varies across areas with varying transmission intensity. Breadth scores were calculated for each child based on the number of antibody levels. Distribution of the breadth of antibody reactivity of malaria-positive children was compared between Kintampo (N=167), Navrongo (N=131), and Accra (N=128).

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Navrongo (P<0.005) and Accra (P<0.005). Similarly, relative proportion of antibodies to Rh2 was significantly lower in children living Accra, compared to children living in Navrongo (P<0.005; Figure 4.6) and Kintampo (P<0.010; Figure 4.6).

Conversely, relative proportion of antibodies to EBA140 RIII-V, EBA181 RIII-V, Rh4.2 and Rh5 decreased with increasing transmission intensity. However, only EBA 140 RIII-V, EBA181 RIII-V and Rh5 showed significant differences when compared across the three sites. Notably, relative proportion of antibodies against PfRh5 decreased with exposure, with children from Accra showing higher relative proportion of antibodies against PfRh5 compared to children living in Kintampo and Navrongo (P<0.001 and P=0.004 respectively; Figure 4.6). Relative proportion of antibodies against EBA140 RIII-V in children living in Accra was also significantly higher compared to children living in Kintampo (P<0.001; Figure 4.6). However, there was no difference in the relative proportion of antibodies to RIII-V of EBA140 in children living in Navrongo compared to children living in Kintampo and Accra (P=0.152 and P=0.114 respectively; Figure 4.6).

Age-adjusted multivariate analysis was performed to determine the relationship between parasite density and transmission intensity with the relative proportion of antibodies against the antigens tested. Relative proportions of EBA140 RIII-V, EBA181 RIII-V and PfRh5 were influenced by endemicity only (F=6.96, P<0.001; F=10.28, P<0.001; F=12.77, P<0.001 respectively; Table 4.2). Relative proportion of EBA181 FL was influenced by both age (F=9.26, P=0.003; Table 4.2) and endemicity (F=12.89, P<0.001; Table 4.2). On the other hand, relative proportions of Rh2 and Rh4 were significantly influenced by parasite density (F=10.22, P=0.002; F=6.17, P=0.002 respectively, Table 4.2) and endemicity (F=3.68, P=0.026; F=4.39, P=0.037 respectively, Table 4.2).
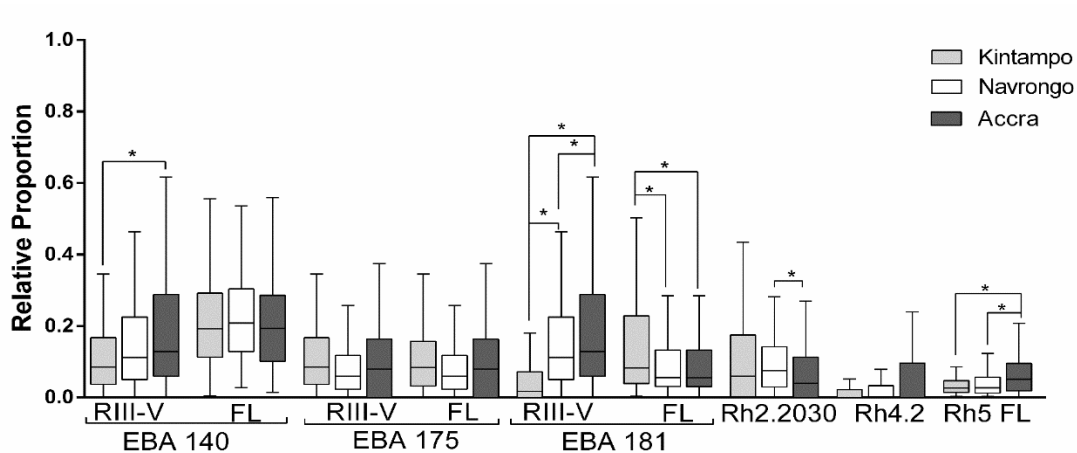
Figure 4.6. Relative proportion of antibodies to recombinant antigens across sites.

Antibody levels against each of the nine invasion antigens for each child was expressed as proportion of the total antibody response to all nine antigens. The box plot represents the group mean proportion for each antigen across sites. Kintampo (N=167), Navrongo (N=131) and Accra (N=128). * indicates P< 0.005 using Mann Whitney U test for comparison. FL=full length

Anti-merozoite antibodies have been shown to significantly inhibit parasite growth in vitro, therefore we examined the relationship between IgG levels against our panel of antigens and parasitaemia. IgG levels of Rh4 were significantly negatively correlated with parasitaemia (ρ=-0.253, P<0.005 Table 4.3).

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Table 4.2. Multiple linear regression analysis of factors associated with relative proportion of anti-merozoite antibody levels in malaria-positive children

| | EBA140 | | | | EBA175 | | | | EBA181 | | | | Rh2 | | Rh4 | | Rh5 FL | |
| | RIII-V | | FL | | RIII-V | | FL | | RIII-V | | FL | | | | | | | |
	F	P-value	F	P-value	F	P-value	F	P-value	F	P-value	F	P-value	F	P-value	F	P-value	F	P-value
Age	1.62	0.203	1.55	0.214	0.14	0.704	3.05	0.082	1.70	0.193	**9.26**	**0.003#**	0.14	0.705	0.21	0.651	1.25	0.265
Parasite Density	0.08	0.774	2.36	0.125	2.22	0.137	**9.01**	**0.003#**	1.29	0.257	1.44	0.231	**10.22**	**0.002#**	**6.17**	**0.002#**	0.06	0.807
Site	**6.96**	**0.001#**	1.09	0.336	1.59	0.205	1.22	0.297	**10.26**	**<0.001#**	**12.89**	**<0.001#**	**3.68**	**0.026**	**4.39**	**0.037**	**12.77**	**<0.001#**

Table summarizing the multiple linear regression analyses. Antibody levels were the outcome variables, while age, parasitaemia, and transmission intensity (sites) were the predictor variables. F = F-statistic. Statistically significant predictors are shown in bold face.

[#]Significant after Bonferroni's P value adjustment. FL=full length.

Table 4.3. Correlation between IgG levels and parasitaemia in malaria-positive Ghanaian children

	Correlation Coefficient (ρ)	P-value
EBA140 RIII-V	-0.081	0.104
EBA175 RIII-V	-0.110	0.027
EBA181 RIII-V	-0.070	0.159
Rh2.2030	0.057	0.252
Rh4.2	-0.253	0.001
EBA140 FL	-0.109	0.029
EBA175 FL	0.051	0.306
EBA181 FL	-0.104	0.036
Rh5 FL	-0.164	0.001

ρ (rho) represents Spearman's rank correlation coefficient. P-values were calculated using Spearman's rank correlation. $P \leq 0.05$ was considered statistically significant. Statistically significant correlations with $\rho \geq \pm 0.25$ are shown in bold face. FL=full length

4.4.7.   Co-acquisition of antibody responses to multiple antigens

Significant correlations were observed between antibody levels to majority of merozoite antigens tested in this study, suggesting that these antigens may be co-acquired (Table 4.4). However, there was variation in the strength of correlation between any two antigens. Strong significant correlations existed between full length and fragments EBA antigens (EBA140, R=0.42, P<0.001; EBA175, R=0.51, P<0.001; EBA 181, R=0.77, P<0.001; Table 4.4). EBA antigens were also strongly correlated with one another, but showed weak correlation with Rh antigens. Rh5 and Rh2 were weakly correlated, whilst Rh4 showed no significant relationship with other Rh antigens.

Table 4.4 Co-acquisition of antibodies against EBA and Rh proteins.

	EBA140 RIII-V		EBA175 RIII-V		EBA181 RIII-V		Rh2-2030		Rh4.2		EBA140 FL		EBA175 FL		EBA181 FL	
	R	*P* value	**R**	*P* value	**R**	*P* value	**R**	*P* value	**R**	*P* value	**R**	*P* value	**R**	*P* value	**R**	*P* value
EBA175 RIII-V	**0.417**	**>0.001**														
EBA181 RIII-V	**0.381**	**>0.001**	**0.498**	**>0.001**												
Rh2-2030	0.099	0.042	0.231	>0.001	0.172	>0.001										
Rh4.2	0.237	>0.001	**0.282**	**>0.001**	0.155	0.001	0.032	0.510								
EBA140 FL	**0.420**	**>0.001**	**0.604**	**>0.001**	**0.552**	**>0.001**	**0.379**	**>0.001**	0.161	0.001						
EBA175 FL	**0.250**	**>0.001**	**0.509**	**>0.001**	0.232	>0.001	**0.557**	**>0.001**	0.123	0.012	**0.486**	**>0.001**				
EBA181 FL	**0.259**	**>0.001**	**0.416**	**>0.001**	**0.772**	**>0.001**	**0.312**	**>0.001**	0.054	0.266	**0.640**	**>0.001**	**0.266**	**>0.001**		
Rh5 FL	0.101	0.038	0.227	>0.001	0.123	0.012	0.160	0.001	0.080	0.100	**0.332**	**>0.001**	0.245	>0.001	**0.281**	**>0.001**

R represents Pearson's correlation coefficient. P-values were calculated using Pearson's correlation. P≤0.05 was considered statistically

significant. Statistically significant correlations with $\rho \geq \pm\, 0.25$ are shown in bold face. FL=full length

4.5. Discussion

Understanding humoral immunity against merozoite antigens may hold the key to the discovery and development of an effective strain transcending vaccine. Recent studies investigating antibody levels to multiple antigens have shown that breadth of antibody reactivity, rather than immunity to a single antigen, is associated with reduced risk of clinical malaria and is a better correlate of protective immunity (Osier *et al.*, 2014b). This has necessitated a shift of focus from studying antibody levels to these antigens individually, to studying them in relation to each other. A recent study by McCallum and others (McCallum *et al.*, 2017) examining acquisition of antibodies against multiple antigens showed that patterns of antibody acquisition are antigen specific, but highlighted the need for studies that allow comparison of antibody response across a variety of age groups and malaria transmission levels. In this study, we compared humoral responses to merozoite antigens, determine the absolute and relative levels of antigen-specific responses, and assessed the breadth of these responses in children with malaria in three areas with varying intensity.

Breadth of antibody reactivity has been shown to be age-dependent (Nogaro *et al.*, 2011) and positively correlated with multiclonality of infection (Rono *et al.*, 2013). Our data confirms that breadth is strongly predicted by age. However, we also show evidence that transmission intensity significantly influences the breadth of antibody reactivity shown by children with malaria in Ghana, independent of the effect of age.

In the present study, we did not observe a correlation between parasitaemia and antibody levels to any of the antigens with the exception of levels to Rh4, that was weakly correlated with parasitaemia. Peculiarly, antibody levels to Rh4 did not correlate with antibodies to any other antigen tested. Previous studies conducted in Papua New Guinea using the same

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antigens observed an association between parasitaemia and antibody levels, and a similar trend was observed in Nigeria (Ahmed Ismail *et al.*, 2014, Reiling *et al.*, 2012, Richards *et al.*, 2010). However, there were no correlations observed between antibody levels to these antigens and concurrent parasitaemia in Uganda (Ahmed Ismail *et al.*, 2013). In our study, parasite density was not a predictor of breadth of antibody reactivity, though we observed significant differences in parasite density across transmission areas. These results support previous observations that antibody levels are more likely as a result of cumulative exposure rather than a single clinical episode of malaria(Doolan *et al.*, 2009).

Humoral immunity, an integral part of the immune response against malaria, is acquired slowly after multiple episodes of clinical malaria (Marsh and Kinyanjui, 2006). As we observed that the endemic site was a significant predictor of antibody levels, we expressed antibody levels to each antigen relative to the sum of antibody levels to all antigens for each child in order to standardize and compare more accurately across the three endemic sites. We observed that relative antibody levels of EBA181, EBA140 and Rh5 differed significantly between the three endemic areas, with the lowest endemic area showing higher levels compared to the highest endemic area. The opposite was true for EBA181 RIII-V and Rh2.

Chronic malaria infection is associated with an increased population of *falciparum*-specific atypical memory B cells that do not differentiate to form antibody secreting cells (Weiss *et al.*, 2009). Expansion of atypical memory B cell populations has also been positively correlated to transmission intensity and parasite infection (Illingworth *et al.*, 2013). Furthermore, studies in HIV have shown that atypical memory B cells are prone to anergy and apoptosis that may lead to low plasma concentrations of antibodies (Hu *et al.*, 2015,

Moir *et al.*, 2008). In malaria, it is unclear whether the expansion of atypical B cells is limited to specific antigens, contributes to the differing rates of antigen-specific antibody acquisition and the delayed acquisition of protective immunity, and plasma concentrations of antibodies (McCallum *et al.*, 2017). Our recent studies within this population has revealed that malaria among children in Kintampo and Navrongo is likely chronic, with majority of children tolerating high parasite densities before succumbing to clinical disease (Ademolue *et al.*, 2017, Mensah-Brown *et al.*, 2017). and hence they may have a larger subset of atypical B cells compared to children living in Accra. The conversion of B cells to anergic or atypical B cells may be limited to specific antigens or may occur at different rates. Further studies that examine the effect of the expansion of atypical B cell on antibody levels are required.

As stated above, we observed that relative antibody levels to full length EBA181 decreased with increasing transmission intensity, while the opposite was true for EBA181 RIII-V. While these are fragment and full length recombinant proteins of the same antigens, the full length antigen may have more immunological epitopes which may account for the differences observed

Taken together, our data reveals that patterns of antibody responses against invasion proteins are both antigen and exposure dependent. We further show that breadth of antibody response is dependent on exposure and age. Deeper understanding of how these factors interplay is needed to understand acquisition of malaria immunity, and the subsequent development of a vaccine.

CHAPTER FIVE

5.0 Paper 3: Levels and breadth of *Plasmodium falciparum*-specific antibody responses in semi-immune adults and their relationship with growth inhibitory activity in vitro.

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Keywords: *Falciparum* malaria, humoral immunity, growth inhibitory activity

5.1. Abstract

Background: *Plasmodium falciparum* causes the majority of morbidity and mortality associated with malaria. Erythrocyte invasion is a crucial step in the life cycle of the pathogen. Several antigens expressed at this stage are the targets of immunity and have been shown to be associated with protection. Antibody levels, antigen specificity and functionality of antibodies to key invasion ligands were evaluated in adults living in a holoendemic area.

Methods: Antibodies against erythrocyte binding antigen (EBA) and reticulocyte-binding homologue (Rh) proteins were detected in plasma samples from adults living in a high transmission area with no recent history of clinical malaria and purified immunoglobulin G (IgG) fractions from these samples were tested in growth inhibition assays against multiple parasite lines.

Results: Different parasite isolates showed varying sensitivities to individual purified IgG fractions, ranging from 40-150% invasion efficiency compared to the uninhibited control. Age was not a predictor of breadth in our sample population.

Conclusion: Total IgG fractions from semi-immune adults show varying growth inhibitory activity against different parasite lines, while some IgG fractions seemed to enhance parasite growth. Growth inhibitory activity was significantly associated with breadth of antibody reactivity, demonstrating the need for careful selection of antigens to include in a potential blood stage vaccine.

5.2. Introduction

According to current estimates by the WHO, there are more than 200 million cases of malaria annually, resulting in approximately half a million deaths (World Health Organization., 2017), the majority being caused by *Plasmodium falciparum*. Approximately 90% of malaria cases occur in sub-Saharan Africa, with at least 80% of deaths occurring in this region, affecting mainly children under the age of five years and pregnant women (World Health Organization., 2017). The clinical symptoms of malaria occur as a result of the erythrocytic stage of the life cycle of the parasite, due in part to inflammatory immune responses. The development of a blood stage vaccine could potentially reduce the incidence of malaria. However, the development of such a vaccine is wrought with many challenges, including the complexity of the immune response and the lack of knowledge of the exact immune mechanism for parasite neutralization. The large number of parasite antigens to which the immune system is exposed, coupled with antigenic variation and polymorphism makes the identification of robust targets of immunity against malaria difficult (Beeson *et al*., 2016).

Previous studies have shown protective associations of antibodies against several merozoite antigens (Fowkes *et al*., 2010). Recently, antibody reactivity has been assayed against larger panels of recombinant antigens, which have identified new associations with protection (Dent *et al*., 2015, Osier *et al*., 2014). Erythrocyte binding antigens (EBA) and reticulocyte binding-like homologue proteins (Rh), which play an essential role in erythrocyte invasion (Cowman *et al*., 2012, Cowman *et al*., 2017), are important targets of immunity (McCarra *et al*., 2011, Persson *et al*., 2013, Tran *et al*., 2014), and have been evaluated as priority candidate antigens for developing a blood-stage vaccine (Richards *et al*., 2013).

Antibodies against EBA and Rh proteins have also shown ability to inhibit parasite growth in vitro (Bustamante *et al*., 2013, Lopaticki *et al*., 2011), supporting the importance of these antigens as targets of immunity. Passive transfer of purified immunoglobulins from malaria-exposed adults to children (Cohen *et al*., 1961, Bouharoun-Tayoun *et al*., 1990) demonstrated the ability of antibodies to clear parasitaemia and mitigate the symptoms associated with malaria, so, purified immunoglobulins from adults may be useful in studying the targets of invasion inhibitory antibodies. Therefore, we explore the relationship between growth inhibitory activity of purified IgG from semi-immune adults and the antibody reactivities to EBA and Rh antigens in semi-immune adults living in an endemic area of Ghana.

5.3. Methods

5.3.1. Study sites and sampling

After obtaining informed written consent, plasma samples were collected from 50 male adults above the age of 18 years. Participants had been living in the vicinity for more than 6 months, and had not had a clinical episode of malaria in at least one year. Kintampo is in the middle belt of Ghana, which is holoendemic for malaria with an entomological inoculation rate of over 250 infective bites per person per year (Asante *et al.*, 2011).

5.3.2. Recombinant antigens

A recombinant antigen panel based on sequences of key merozoite invasion ligands was used for assays to study antibodies in plasma samples. Five of the antigens, Regions III-V (RIII-V) of EBA140 (3D7; amino acids 770-1064), RIII-V of EBA175 (3D7; amino-acids, 761-1298), RIII-V of EBA181 (3D7; amino-acids 769-1365), PfRh2.2030 (3D7; amino acids 2027 to 2533, common region for both PfRh2a and PfRh2b) and PfRh4.2 (3D7; amino acids 1277–1451), were expressed in *Escherichia coli* cells and purified using glutathione-agarose beads (Reiling *et al.*, 2010, Reiling *et al.*, 2012, Richards *et al.*, 2010). Full-length (FL) ectodomains of EBA140, EBA175, EBA181 and PfRh5 were also expressed as biotinylated proteins in human embryonic kidney (HEK) 293E cells and purified by nickel affinity chromatography (Crosnier *et al.*, 2013).

5.3.3. Enzyme Linked Immunosorbent Assays

Enzyme linked immunosorbent assays (ELISAs) were performed to determine the relative levels of antigen-specific antibodies in plasma samples. Antibody responses to the GST-tagged antigens (EBA140 RIII-V, EBA175 RIII-V, EBA181 RIII-V, PfRh2.2030, and PfRh4.2) were measured as previously described (Polley *et al.*, 2003), with slight

modifications. Briefly, wells of flat bottom 96-well plates (Immulon 4HBX, Thermo Scientific) were coated at 0.50 µg/mL concentration of individual recombinant antigens (GST-tagged antigens) in 50 µL of coating buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, pH 9·3) and incubated overnight at 4°C. The plates were washed with phosphate buffered saline (PBS) containing 0·05% v/v Tween 20 (PBS-T) and blocked for 3 hours with 1% skimmed milk (Marvel™ UK, London, UK) in PBS-T at room temperature. Plates were washed prior to addition of 50 µL of plasma, diluted 1/500 in blocking buffer and overnight incubation at 4°C. The plates were washed once more, incubated with 100 µL of horseradish peroxidase-conjugated rabbit anti-human IgG (at a dilution of 1/15,000) (Dako UK Ltd, Ely, UK), washed again, before the addition of 100 µL of tetramethylbenzidine substrate (TMB) solution. After incubation for 20 minutes, reactions were stopped using 50 µL of 0.2 M sulphuric acid, and optical densities were measured at 450 nm in a microplate reader (Bio-Rad iMark, Hertfordshire, UK). All plasma samples were assayed in duplicates and blank wells were set up using GST only.

ELISAs using biotinylated antigens were done as previously described (Osier *et al.*, 2014b), with minor modifications. Streptavidin-coated 96-well ELISA plates (NUNC, Denmark) were washed with PBS-T and blocked for 30 minutes with PBS containing 0.5% bovine serum albumin (BSA) at room temperature, prior to coating with individual recombinant antigens at 0.50 µg/mL concentration. Antigen-coated plates were incubated at room temperature for 45 minutes and washed with PBS-T. Plasma samples were added at 1:1000 dilution in blocking buffer and incubated overnight at 4°C. The plates were washed and incubated with 100 µL of horseradish peroxidase-conjugated rabbit anti-human IgG (at a dilution of 1/15,000) (Dako UK Ltd, Ely, UK). The plates were washed once again prior to addition of TMB substrate, that which incubated for 20 minutes. Reactions were stopped and

optical densities were measured as described above. Antibody reactivities of 20 nonimmune individuals (malaria non-exposed Europeans) were tested as negative controls in all assays. Antibody reactivity of a sample was considered positive if the OD was greater than mean +3 standard deviations of these control individuals.

5.3.4. Purification of Total IgG

Total IgG was purified from 55 plasma samples using a protein G affinity column (GE Healthcare, Uppsala, Sweden), in accordance with the manufacturer's instructions on the AKTA Pure 25 protein purification system (GE Healthcare, Uppsala, Sweden). Briefly, the column was equilibrated using five column volumes of 20mM sodium phosphate buffer (pH 7.00), after which the diluted plasma samples were injected on the column. The column was washed thoroughly with the equilibration buffer before eluting with 0.1M Glycine buffer (pH 2.8). Purified IgG fractions were collected and visualized on 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel.

5.3.5. Native and Non-native SDS-PAGE

Proteins were solubilized in SDS-PAGE sample buffer containing 1% SDS, 66 mM Tris–hydrochloric acid (HCL), 1% Glycerol, 0.7% Bromophenol Blue and 10 mM β-mercaptoethanol and boiled at 100^OC for 10 minutes. Samples in non-reducing conditions were solubilized in the sample buffer without 10 mM β-mercaptoethanol. Five microliters of each sample was loaded in each lane on a 12% SDS polyacrylamide gel The protein bands were visualized by staining with 0.1% Coomassie Brilliant Blue R-250 in 10% acetic acid and 30% methanol.

5.3.6. Protein Quantification

Protein concentration of IgG fractions was determined using the Pierce bicinchoninic acid (BCA) Protein Assay Kit, according to manufacturer's instructions Thermo Scientific, Waltham, Massachusetts, US). Purified IgG fractions were diluted (1:100) and mixed with BCA reagent in a 1:8 reaction mixture on a microtiter plate. The plate was then incubated at 37ºC for 30 minutes and absorbance read at 562 nm on a Varioskan™ LUX multimode microplate reader (Thermo Scientific, Waltham, Massachusetts, USA).

5.3.7. MSP-2 Allele Typing

Two allelic families (FC27 and IC3D7) central polymorphic region of msp2, nested PCR was performed using family specific primers (Snounou *et al.*, 1999). All amplification reactions were carried out in a final volume of 15 μl. The outer PCR reaction mix contained 200 nM dNTP, 2 mM MgCl2. 133 nM of each primer, and 0.5 unit of One Taq DNA polymerase (New England BioLab, UK) in addition to 4 μl (about 0.25 μl of whole blood) of genomic DNA (gDNA) template. In the nested reaction, 0.5 μl of the outer PCR product was used as template in a PCR reaction mixture containing 200 nM dNTP, 1.8 mM MgCl2. 200 nM of each primer and 0.5 unit of One Taq DNA polymerase. Each amplification profile consisted of initial denaturation at 94 °C for 3 min, followed by 30 cycles at 94 °C for 1 min; 50–59 °C (depending on the primer pair annealing temperatures) for 35 s, and 68 °C for 2.5 min; with final extension at 68 °C for 3 min. PCR products were separated using 2% ethidium bromide-stained agarose gels respectively and visualized under UV illumination.

5.3.8. Invasion assays

Erythrocyte invasion assays were set up using schizont stage parasites according to methods described by Theron *et al.* (2010), with slight modifications. Parasite cultures with parasitaemia between 1-5% of predominantly schizont stage parasites (>90%) were used for invasion assays. The target erythrocytes used in the assays were stained with 5[6] Carboxyfluorescein diacetate, succinimidyl ester (CFDA-SE, Sigma Aldrich, St. Louis, MO USA) to differentiate them from the uninfected erythrocytes in the parasite inoculum, using previously described procedures (Theron *et al.*, 2010). Parasitized erythrocytes were incubated with CFDA-SE-stained erythrocytes at 2% haematocrit and purified human IgG fractions at 5mg/mL in a 96-well titre plate. The plate was incubated at 37ºC for approximately 20 hours in a gas mixture of 2% O_2, 5.5% CO_2, and 92.5% N_2. All experiments were set up in duplicate wells. Hoechst 33342 (Invitrogen, Eugene, OR, USA) was used to stain parasite DNA to differentiate parasitized erythrocytes from uninfected erythrocytes. Invasion levels were then determined using flow cytometry to analyse fluorescence of the erythrocytes.

5.3.9. Data Acquisition by flow cytometry

A 1:20 dilution of each well was done by adding 10μL aliquots of culture from each well to 190μL of FACS Flow (BD Biosciences) in round bottom 96-well plate. The samples were acquired on a BD Fortessa X-20 flow cytometer (BD Biosciences). CFDA-SE was excited by a blue laser and detected by a 530/30 filter. Hoechst 33342 was excited by a UV laser and detected by a 450/50 filter. BD FACS Diva® was used to determine invasion levels for 50,000 cells per well.

5.3.10.   Statistical Analyses

Statistical analyses were performed with Minitab. Students t-test was used to assess the effect of purified IgG fractions on parasite invasion efficiency. Pearson and Spearman's rank correlation coefficients were used to assess the association between two continuous variables. The breadth of antibody responses was assessed by assigning each individual a breadth score (between 0 and 9) depending on the number of antigens to which they tested positive. Multivariate regression analyses were carried out to determine which antigens predicted inhibitory activity. For all analyses, $P<0.05$ was considered statistically significant.

5.4. Results

5.4.1. Antibody responses to merozoite antigens measured by ELISA

IgG responses to merozoite antigens EBA140 (RIII-V and full length (FL)), EBA175 (RIII-V and full length), EBA181 (RIII-V and full length) PfRh2.2030, PfRh4.2 and full length PfRh5 were evaluated by ELISA. All participants tested were seropositive for at least one antigen. Median antibody ODs to EBA antigens were 0.325 [IQR 0.240-0.519], 0.143 [IQR 0.076 - 0.388], 0.314 [IQR 0.162-0.450], 0.274 [IQR 0.135-0.768], 0.053 [IQR 0.000-0.202], 0.161 [IQR 0.056-0.563] for EBA140 RIII-V, EBA140 FL, EBA 175 RIII-V, EBA175 FL, EBA181 RIII-V, EBA181 FL respectively (Figure 5.1). Median antibody ODs to Rh antigens were 0.049 [IQR 0.000- 0.235], 0.001 [IQR 0.000-0.090], 0.049[IQR 0.028-0.086] for PfRh2, PfRh4 and PfRh5 respectively (Figure 5.1). Seroprevalence of antibodies to EBA140 RII-V, EBA140 FL, EBA175 RIII-V, EBA175 FL, EBA181 RIII-V, EBA181 FL, Rh2, Rh4, and PfRh5 was 96%, 42%, 78%, 60%, 42%, 62%, 42%, 28%, and 26% respectively. Antibody response to full length and fragment antigens of EBA175 and EBA140 were strongly correlated with one another (R=0.779, *P*<0.001; R=0.641, *P*<0.001

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respectively, while responses to full length and fragment antigens of EBA181 showed significant but weaker correlation (R=0.354, *P*=0.012). No association was found between age and antibody ODs for any of the antigens tested.
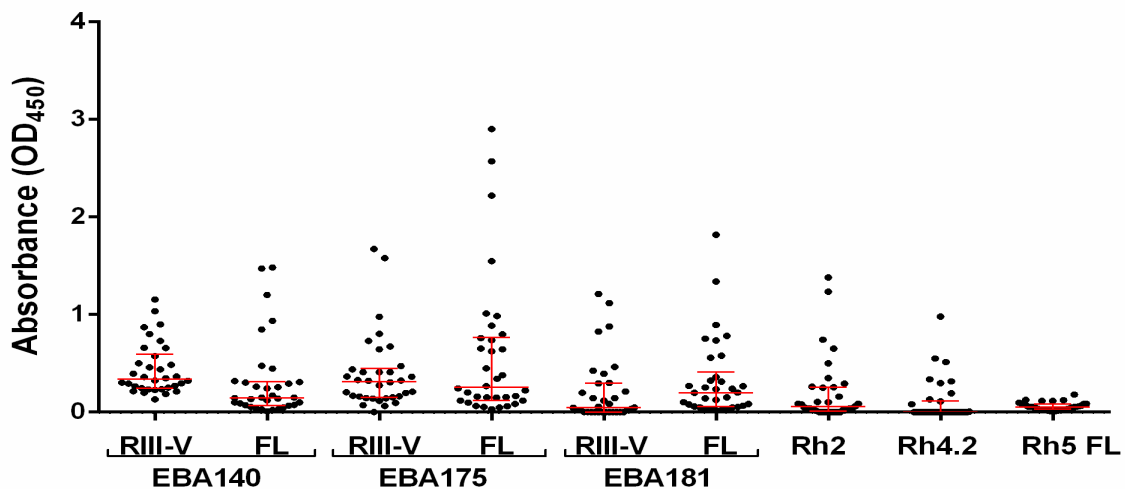
Figure 5.1. Antibody ODs of adults from Kintampo against EBA and Rh antigens.

Enzyme linked immunosorbent assays were conducted using plasma collected from 50 semi-immune adults residing in Kintampo. Data is presented as a dot plot. Lines represent median. Error bars represent IQR. FL=full length

5.4.2. Invasion inhibitory activity of purified IgG fractions from adults

To assess the relationship between antibody responses to EBA and Rh antigens and parasite inhibition, *in vitro* growth inhibitory assays were performed using purified IgG from adults against four long-term laboratory-adapted strains (3D7, W2mef, K1 and GB4) and three local clinical isolates from Kintampo (EIMK084, EIMK239, and EIMK244). There was sufficient purified IgG amount from 36 of the donors for these assays of inhibition of erythrocyte invasion across all seven parasite lines. On average, purified IgG from individual donors showed ability to inhibit parasites on average by 15%, ranging between 10-40% compared to the uninhibited control cultures (Figure 5.2). There were apparent differences in the levels of inhibition measured for the assays performed on different parasite lines (Supplementary Figure 5.2). For example, the laboratory isolate 3D7 was significantly inhibited by IgG from donors EIMKB33 and EIMKB20 compared to the non-immune

control ($P<0.05$, Supplementary Figure 5.2A-B), whereas the W2mef strain was inhibited by IgG from most individuals, but IgG had less inhibitory effects in the assays performed on parasite isolates GB4 and K1 (Supplementary Figure 5.2C-D). In assays using the three local parasite isolates, there was more IgG inhibition seen against isolates EIMK239 than isolate EIMK084 and EIMK244 ($P<0.05$, Supplementary Figure 5.2E-G). It is unknown whether these differences are due to actual variation among the isolates, as each one was not assayed with multiple independent biological preparations. For the measurement of invasion inhibition overall, the mean of the inhibitory activity across all seven isolates was considered in the following analysis.
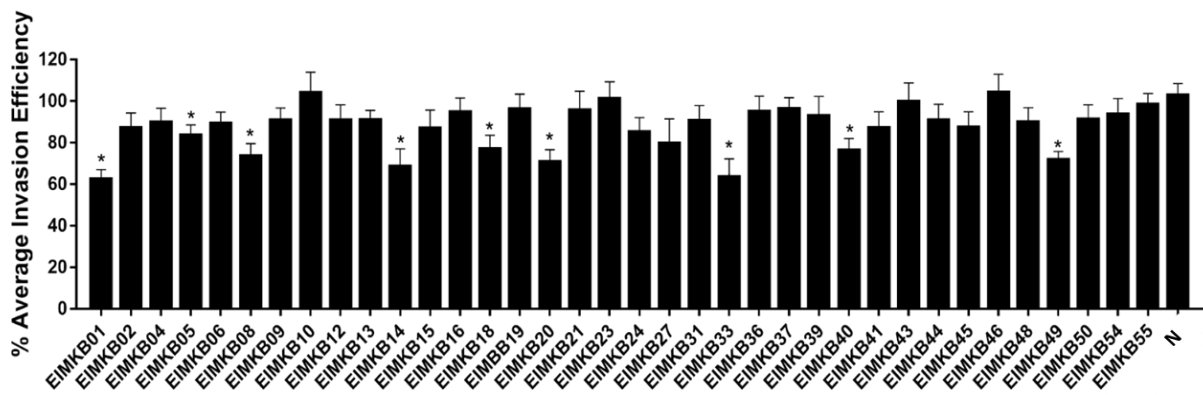
Figure 5.2. Average growth inhibitory activity of purified IgG from adult donors against seven P. falciparum isolates. Growth inhibition assays were set up in duplicate in 96 well plates to test the inhibitory activity of 36 purified IgG preparations (at 5 mg/ml) from semi-immune adults living in Kintampo. Four laboratory strains 3D7, W2Mef, K1 and GB4 and three clinical isolates (E) EIMK084, EIMK239, EIMK244. were tested. Parasitaemia was determined by flow cytometry using BD FACS Fortessa. Data are presented as a percentage of invasion efficiency in the uninhibited control. Error bars represent SEMs. *Invasion inhibition by purified IgG is statistically significant relative to uninhibited control (P< 0.05). N=Non-Immune European.

5.4.3.   Breadth of antibody reactivity is correlated with invasion inhibition.

Breadth of antibody reactivity has previously been shown to be a predictor of protective immunity against malaria. Therefore, we examined the relationship between breadth and growth inhibitory activity. Breadth of antibody reactivity was calculated based on seropositivity to all nine antigens. The highest proportion of plasma samples were seropositive for three antigens (22%), whilst less than 5% recognized 8 antigens (Figure 5.3).

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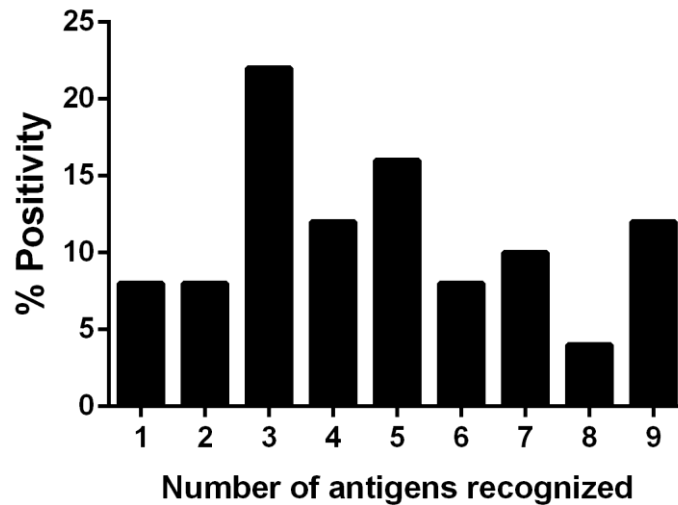
Figure 5.3. The breadth of response of semi-immune adults from Kintampo against multiple invasion antigens. Breadth scores were calculated for each adult based on the number of positive antibody responses. Distribution of the breadth of antibody reactivity of malaria-positive children is shown. Bars represent the proportions of individuals with breadth scores from 1-9.

Whilst the breadth of antibody reactivity was not correlated with age (Spearman's rho=-0.011, P=0.943; Figure 5.4A), a significant association was observed between breadth of antibody reactivity and invasion inhibitory activity (Spearman's rho=0.437, P=0.0097; Figure 5.4B). Antibody OD levels for full length and RIII-V of EBA140, full length and RIII-V of EBA175, RIII-V of EBA181, Rh2, Rh4 and PfRh5 were significantly correlated with invasion inhibition (P<0.05 for all analysis; Figure 5.5)
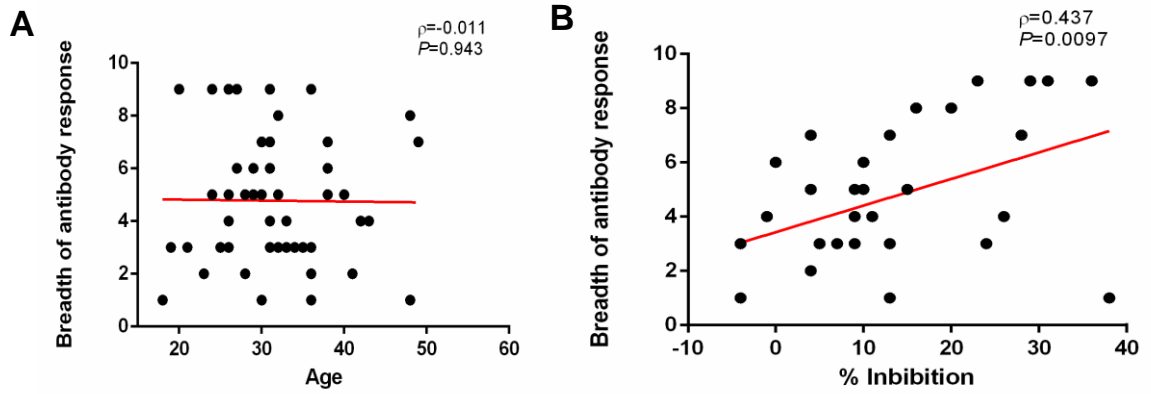
Figure 5.4. Breadth of antibody reactivity is associated with growth inhibition. Dot plot showing correlation between breadth of antibody reactivity and mean inhibition of purified IgG fractions observed when tested against four laboratory strains (3D7, W2mef, K1 and GB4) and three clinical isolates (K244, K239 and K084).
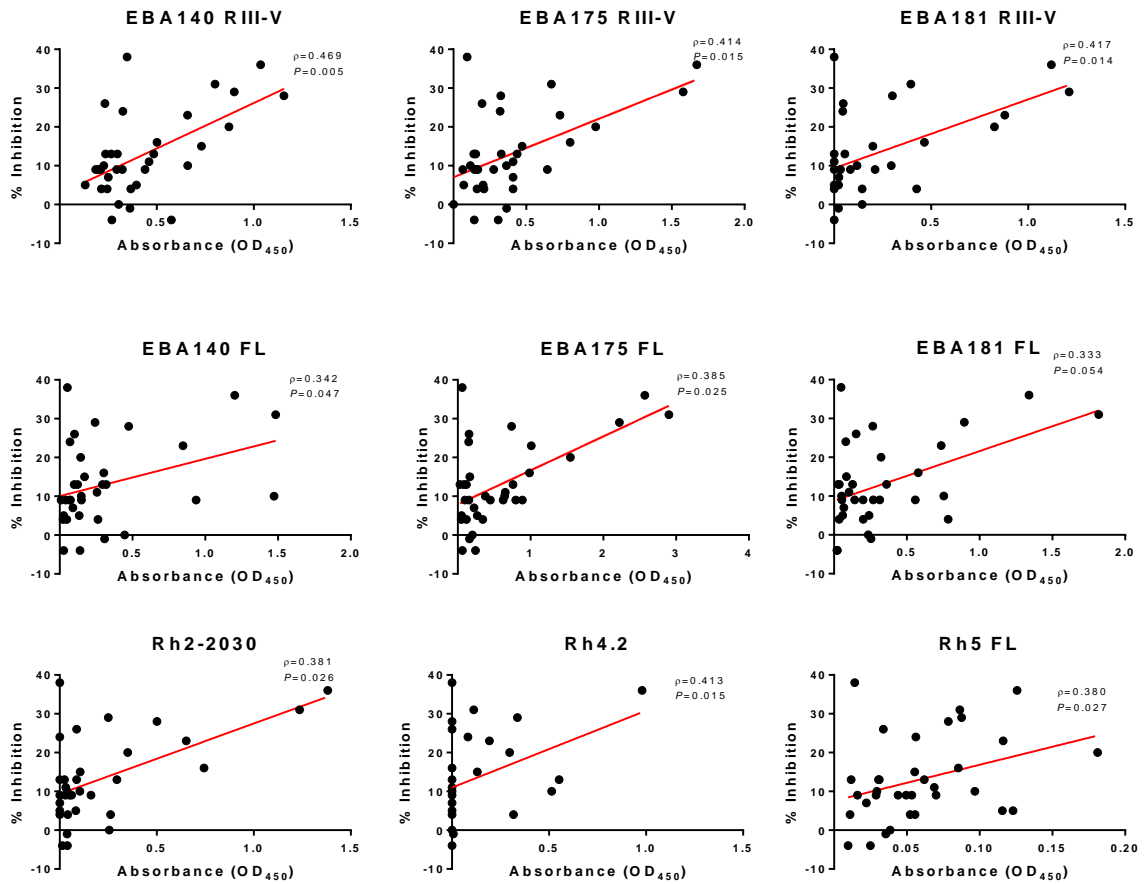
Figure 5.5. Correlation between ELISA OD measures of antibody levels and mean inhibition by purified IgG. Average invasion inhibition of seven   parasite lines by donor IgG was correlated with reactivity to EBA and Rh antigens. Correlations of P<0.05 were considered statistically significant by the Spearman test**.**

5.5. Discussion

Previous studies have shown that antibodies against EBA and Rh proteins have greater inhibitory effect against parasites in culture when used in combination (Lopaticki *et al.* (2011), supporting the idea that antibodies against multiple antigens can act in combination to provide protection against malaria. In this study we sought to examine the correlation between naturally acquired antibody levels to EBA and Rh recombinant antigens and inhibitory activity against parasites in culture.

Growth inhibitory activity of purified IgG fractions from a panel of adult donors living in a malaria endemic were tested against seven parasite lines. Similar to what has previously been reported with IgG from immune donors (Brown *et al.* (1982), varying rates of invasion were observed compared to the untreated controls. Purified IgG from some samples did seem to enhance the growth of some parasite lines *in vitro*, and such enhancement of growth has previously been reported in other studies. Some antibodies to recombinant antigens have been shown to enhance merozoite invasion *in vitro* (Franzén *et al.*, 1989). and it has also been reported that pooled IgG from West African donors enhanced parasite growth *in vitro* (Bouharoun-Tayoun *et al.*, 1990). Another study showed that the majority of IgG preparations from Kenyan donors tested enhanced parasite growth by varying degrees (Shi *et al.*, 1999). It is difficult to determine what could account for this enhancement of growth, but antibody-mediated enhancement of infection has been reported in other pathogens such as HIV and dengue fever (Gorlani and Forthal, 2013, Halstead, 2014). Complement-mediated enhancement of parasite growth has recently been reported in *P. falciparum* (Biryukov *et al.*, 2016). As these assays were conducted in in the absence of complement,

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further studies are required to determine possible mechanisms of antibody-dependent enhancement of growth, if any exist.

The present analysis showed that breadth of antibody reactivity to multiple antigens was associated with inhibitory activity of purified IgG, and the individual specific antibody levels were also correlated with inhibition. Osier *et al.* (2008) reported that breadth of reactivity against different merozoite antigens to be associated with reduced risk of malaria in cohort studies in Kenya. A more recent birth cohort in Kenya reported that there was growth inhibitory activity of IgG but that this did not increase with increasing breadth of antibody reactivity by ELISA assays (Murungi *et al.*, 2016). More research is required to test the hypothesis that breadth of antibody reactivity against different antigens is a key determinant of protection against malaria, as suggested by others (Beeson *et al.*, 2016, Bolad and Berzins, 2000, Osier *et al.*, 2008). One study (Iqbal *et al.* (1997) has suggested that parasites grown in the presence of antibodies against two vaccine candidate antigens led to the emergence of clones that showed reduced sensitivity to antibody mediated parasite inhibition. Although such possible selection has not been studied independently, if this effect is real it would indicate the need for a multicomponent vaccine, in order to prevent the emergence of vaccine resistance.

In summary, our data show that IgG from plasma of semi-immune adults show varying growth inhibitory activity against *P. falciparum* in culture, while some IgG preparations seemed to enhance parasite growth. We further show that growth inhibitory activity was significantly associated with antibody levels and breadth of antibody reactivity to different EBA and Rh antigens, supporting the idea that different antigens could be included in a

potential blood stage vaccine. Further studies are required to identify and prioritize the targets of growth inhibitory antibodies for future vaccine development.

5.6. Supplementary Figures
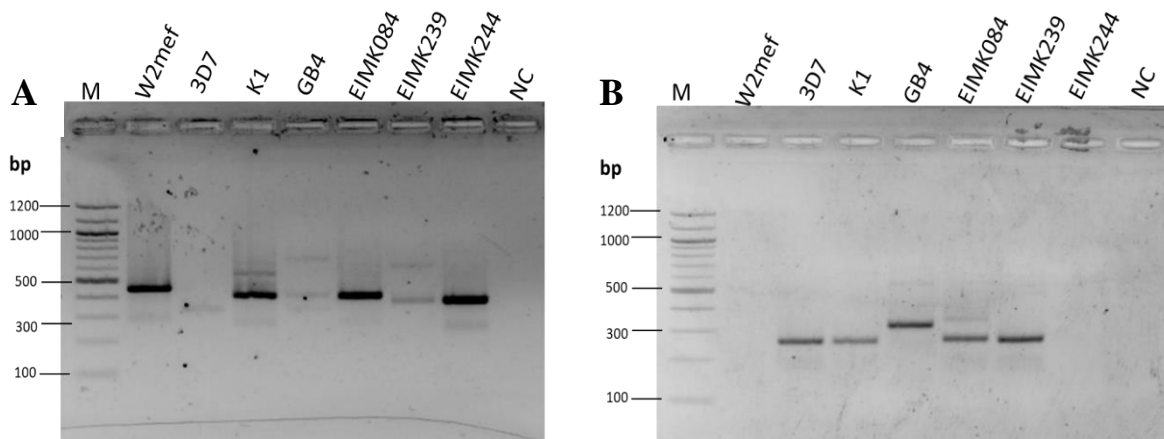
Supplementary Figure 5.1. MSP-2 allele typing of laboratory strain and clinical isolates of *P. falciparum* used for growth inhibitory assays. Laboratory strains and clinical isolates of *Plasmodium falciparum* were typed using (A) FC27 and (B) 3D7/ICI primers. PCR products were run on a 2% ethidium bromide agarose gel and visualized by UV transillumination.

A

3D7

B

W2mef

C

GB4

D

K1
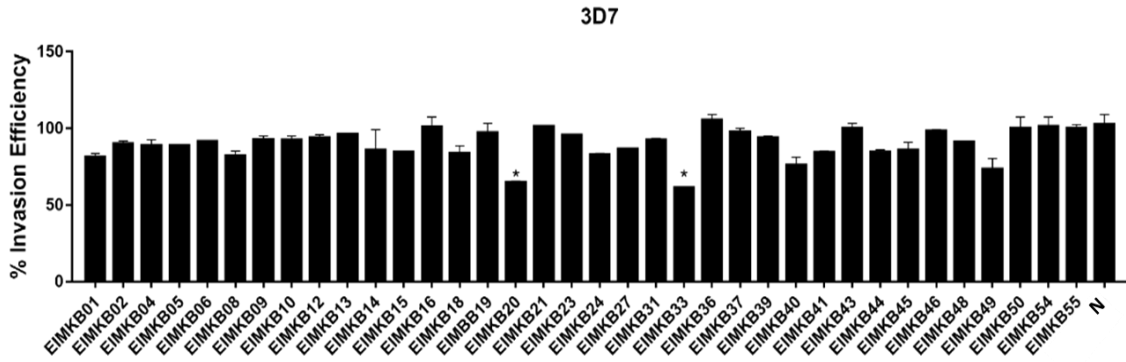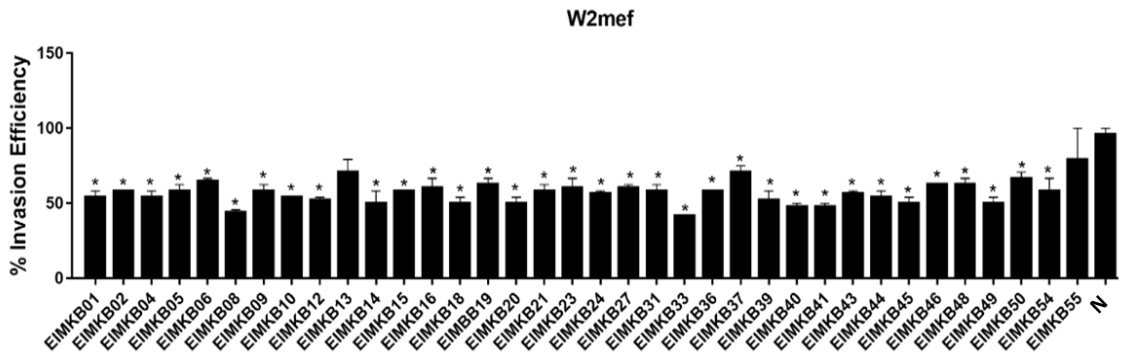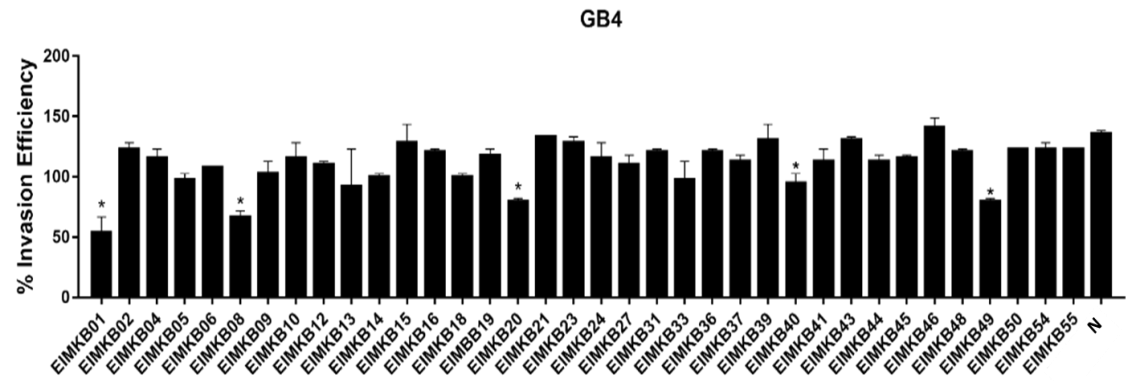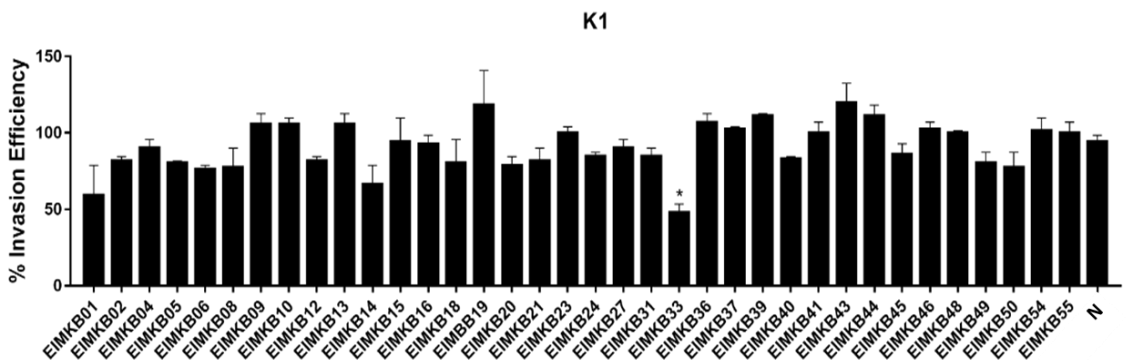
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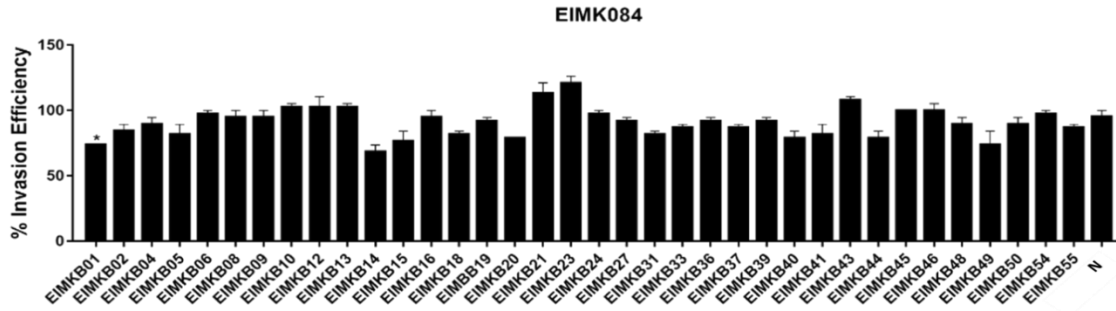
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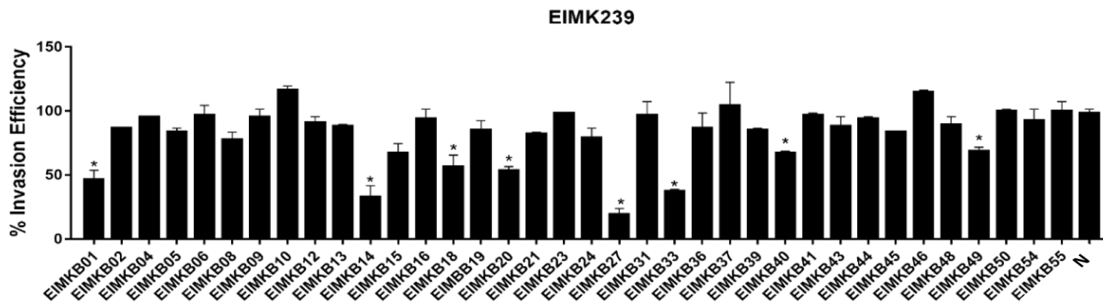
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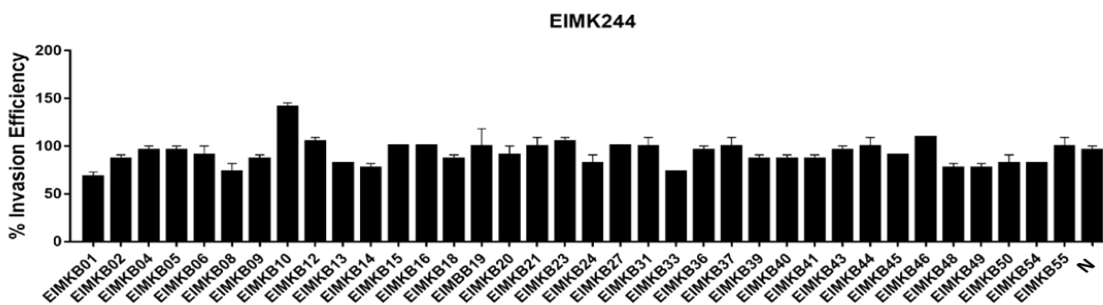
Supplementary Figure 5.2. **Growth inhibitory activity of purified IgG against laboratory strains and clinical isolates.** Growth inhibition assays were set up in duplicate in 96 well plates to test the inhibitory activity of 36 purified IgG preparations (at 5 mg/ml) from semi-immune adults living in Kintampo. Four laboratory strains (A) 3D7, (B) W2Mef (C) K1 and (D) GB4 and three clinical isolates (E) EIMK084, (F) EIMK239 (G) EIMK244 were tested. Parasitaemia was determined by flow cytometry using BD FACS Fortessa. Error bars represent SEMs. *Invasion inhibition by purified IgG is statistically significant relative to uninhibited control. N=Non-Immune European.

CHAPTER SIX

6.0 General discussion and conclusion

Erythrocyte invasion is a crucial point in both the parasite life cycle and the acquisition of immunity. Antibodies that target invasion antigens play an important role in disrupting erythrocyte invasion, making these antigens excellent vaccine candidates. However, functional redundancy, polymorphisms and antigenic variation exhibited by merozoite antigens involved in erythrocyte invasion make it difficult to identify the most important targets of invasion inhibitory antibodies.

Naturally acquired immunity to malaria develops after repeated exposure, and hence the rates of acquisition are higher in people who live in higher endemic areas. From previous evidence, it is clear that protective immunity is targeted at multiple antigens, and antibodies against these antigens may work in combination to achieve protection.

Protective immunity to malaria is determined by the specificity, breadth, magnitude and patterns of responses, which are in turn influenced by multiple factors including age and exposure (Fowkes *et al.*, 2016). As the incidence of malaria continues to decline and people are less exposed to malaria, a deeper understanding of the impact of decreasing transmission on immune responses is needed to guide control efforts. The present study was designed to examine the patterns of responses to invasion antigens in relation to age, exposure and parasite density. Additionally, the relationship between the ability of purified IgG to inhibit parasite growth *in vitro* and the antibody reactivities to EBA and Rh antigens in semi-immune adults living in an endemic area of Ghana was explored.

In order to achieve these objectives, plasma was collected from malaria-positive children aged 2-14 years in three ecologically distinct endemic areas in Ghana, to encompass the spectrum of malaria immunity, from non-exposed to semi-immune children. For the identification of targets of invasion inhibitory antibodies, plasma was collected from 50 adults living in an area of high malaria transmission. This was to ensure that sufficient volume of IgG would be obtained to conduct invasion assays using multiple parasite lines.

Firstly, the impact of the variation observed in transmission intensity on the demographic, clinical, haematological characteristics on our study population was examined. Whilst significant differences were observed in the average parasitaemia of children living in the different endemic areas, higher parasitaemia was not associated with greater severity of malarial anaemia, corroborating what has previously been shown (Novelli *et al.*, 2010, Ong'echa *et al.*, 2006). However, our data showed that sickle cell trait was protective against SMA within our study population. The level of education of the parents and their employment status was associated with the severity of SMA, with children of lower income, less educated parents being more susceptible. These observations have been discussed in detail in chapter 3.

SMA was more common among younger children and children living in areas of high malaria transmission in our study, which may be suggestive of chronic infections. A recent study of our study population revealed that children living in Kintampo had significantly reduced levels of IL-1β, IL-12 and IL-10, compared to children who were living in Accra (Ademolue *et al.*, 2017). As reduced levels of IL-1β, IL-12 and IL-10 are associated with SMA (Kurtzhals *et al.*, 1998, Luty *et al.*, 2000, Ouma *et al.*, 2008, Perkins *et al.*, 2000), the observation that the proportion of children with SMA in Kintampo was significantly higher

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than Accra is consistent with previously published results. Studies have shown that the overall risk of developing SMA reduces with age (Reyburn *et al.*, 2005), which we also observed in our study. Carriers of the sickle cell trait were protected against SMA, which is consistent with previous observations (Amodu *et al.*, 2012, Amoako *et al.*, 2014, Kreuels *et al.*, 2010, Aidoo *et al.*, 2002, Lell *et al.*, 1999).

To achieve our second objective, antibody levels to key invasion antigens were measured using ELISA. Seroprevalence of EBA140 RIII-V was approximately 40% and 96% in symptomatic children and healthy adults respectively. Seroprevalence rates among symptomatic children was lower than in previous reports that found EBA140 RIII-V seroprevalence of 88% and 100% in Uganda and Nigeria, respectively (Ahmed Ismail *et al.*, 2013, Ahmed Ismail *et al.*, 2014). EBA175 RIII-V and EBA181 seroprevalence was between 20-60% in malaria positive children, whilst seroprevalence among adults was 78% and 42% respectively. Seroprevalence of Rh4 and Rh2 was also low with average rates of 30% and 50% respectively. Seroprevalence to majority of the antigens tested was low compared to studies conducted in Papua New Guinea, Nigeria and Uganda that showed seroprevalence rates of nearly 100% (Ahmed Ismail *et al.*, 2013, Ahmed Ismail *et al.*, 2014, Reiling *et al.*, 2010). Seropositivity to Rh5 ranged between 10-20% in our study populations which is in agreement with previous studies (Douglas *et al.*, 2011, Partey *et al.*, 2018, Tran *et al.*, 2014). The difference in seroprevalence observed in our study compared to the rates observed in other studies using the same antigens may be due to differences in the malaria endemicity between the various study sites or the age range of study participants.

Currently, there is no clear understanding of how malaria transmission affects immunity and how much exposure is required for the development of protective immunity (Fowkes *et al.*,

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2016). There are concerns that the decline in malaria transmission would affect the development of immunity, leading to an increase in malaria cases and the number of people at risk of severe malaria. Through the evaluation of immunity to merozoite antigens, in malaria-positive children in areas of Ghana that show difference in transmission intensity, we provide some insights into the differences that may be peculiar to specific transmission settings. Absolute and relative antibody levels were significantly influenced by age and endemicity. The majority of children living in a high transmission setting were seropositive to a larger repertoire of antigens, which may translate into greater growth inhibitory activity since we observed a relationship between breadth of antibody reactivity and growth inhibitory activity. From the present analyses, it may seem antibody responses are antigen and exposure dependent. For some antigens, the relative levels of antibodies appeared to decrease in an exposure dependent manner, an observation which may need to be validated in subsequent studies.

We observed that breadth of antibody reactivity was correlated with parasitaemia. However, the predictors of breadth of antibody reactivity were endemicity and age in children with malaria sampled in our cross-sectional study. Studies conducted in Kenya found an association between breadth of antibody reactivity and parasitaemia with a limited number of antigens (Osier *et al.*, 2008, Rono *et al.*, 2013). Another study conducted in Kenya showed this association was not significant when three or more antigens were assayed (McCallum *et al.*, 2017). This may suggest that increased breadth of antibody reactivity may be more dependent on the number of times individuals are exposed to the parasite, rather than parasitaemia during a single clinical episode.

Antibody levels were weakly or moderately correlated with one another, with the exception of like antigens. This observation is similar to what has previously been observed in another study in Kenya (Osier *et al.*, 2014b). It has been proposed that the differences observed in the rates of acquisition of antibodies to merozoite antigens may be due to differences in the immunogenicity as well as the subcellular localization of the antigens, and that antibody responses are not co-acquired (Ademolue *et al.*, 2017). The rates of decay of merozoite specific antibodies are also not yet fully understood, though evidence from immune-epidemiological studies suggest that antibodies acquired during a malaria transmission season are partially lost over the course of the dry season, when there is little to no malaria transmission. It has also been suggested that the rates of acquisition of antibodies are antigen-specific, and different antigens may elicit antibody secreting cells with different longevity (Hviid *et al.*, 2015, McCallum *et al.*, 2017). These factors may account for the weak correlations observed between antigens in this study. Further studies will be required to ascertain whether the development and sustenance of antibody secreting cells is antigen-specific, and the effect of factors such as transmission and age.

Previous studies have shown that antibodies against invasion antigens have inhibitory activity, and that combinations of these antibodies increase invasion inhibitory activity (Lopaticki *et al.*, 2011), which has significant implications for vaccine design and their prospects as vaccine candidates. Growth inhibitory activity is mediated by IgG and hence purified IgG from semi-immune adults was used to examine the relationship between growth inhibitory activity and the antibody reactivities to EBA and Rh antigens in semi-immune adults living in an endemic area of Ghana. Average invasion efficiency was approximately 30%, which is similar to the inhibitory activity of plasma from healthy adults in Kenya

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against three laboratory strains of *Plasmodium* (Dent *et al.*, 2008). In that study, age was negatively correlated with growth inhibitory activity which was not observed in the present study. Another study in Kenya assessed the relationship between growth inhibitory activity against three plasmodium strains and plasma levels of antibodies to AMA-1, MSP-1$_{19}$, MSP-2, MSP-3 and Rh2 (Murungi *et al.*, 2016). The researchers did not find a significant relationship between growth inhibitory activity and antibody levels to any of the antigens with the exception of MSP-3 (Murungi *et al.*, 2016). In this study, average invasion inhibitory activity against seven parasite lines was significantly positively correlated with antibody reactivity to all EBA and Rh antigens. This variation in results may be due to the difference in the antigens used and notable differences in the two assays, namely the use of dialyzed plasma versus purified IgG and the use of a two cycle assay vs one cycle assay. Additionally, the nature of the antigens and their relative importance to the process of invasion may also account for this observation.

However, in terms of vaccine design, it may not be prudent to put several antigens in a single vaccine as it may affect cost of production. Hence it is still necessary to determine the combination of antigens that is most potent for growth inhibition. Subsequent growth inhibitory assays may need to be conducted to assess the individual contribution of each specific antigen to growth inhibition by depleting antibody concentration using the antigens.

6.1.Conclusions

1. Significant differences were observed in red cell, white cell and platelet indices across the three endemic areas. The proportion of children with malaria anaemia decreased with decreasing transmission intensity.

2. Sickle cell trait was significantly protective against SMA in the most endemic area. The level of education and employment of parents were the most significant predictors of malarial anaemia.

3. Antibody levels to EBA181, Rh2 and Rh4 differed significantly across the three endemic areas. No differences were observed in the absolute levels of the other antigens tested.

4. Relative levels of antibodies to EBA181, EBA140 and Rh5 varied significantly across the three sites. Regression analyses showed that endemic area and age were the most common predictors of relative antibody levels.

5. Low seroprevalence rates to EBA and Rh antigens were observed in Accra compared to Navrongo and Kintampo.

6. Breadth of antibody reactivity correlated with age and parasitaemia. However, the strongest predictors of breadth were age and endemic area.

7. Antibody levels to EBA and Rh antigens were significantly correlated with growth inhibitory activity of IgG from semi-immune adults.

8. Breadth of antibody reactivity was also correlated with growth inhibition.

6.2. Recommendations

The study identified differences in the absolute and relative antibody levels in children with malaria across the three endemic areas. Additional experiments are recommended to determine the cause of the observed variation. Longitudinal studies that would investigate the acquisition of antibodies, as well as the development and maintenance of B cell memory. It may also be important to examine the decay rates of antibodies to these antigens and the rates of conversion to antibody secreting cells, and their effect of plasma antibody concentrations.

We observed that breadth of antibody reactivity was associated with growth inhibitory activity of purified IgG from semi-immune adults. Further experiments should include morbidity checks, to further determine the relationship between growth inhibitory activity and the risk of clinical disease.

In order to identify the important targets of invasion inhibitory antibodies, experiments involving competitive inhibition of sera with purified proteins of the invasion ligands should be conducted.

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Appendix

Table A 1. Antibody levels of children living in three endemic areas against EBA and Rh antigens measured as ODs.

Sample	Study Site	Age (years)	Sex	Parasite Density (parasites/μL)	Antibody levels (Absorbance$_{450}$)								
					EBA140	EBA175	EBA181	Rh2.2030	Rh4.2	EBA140 FL	EBA175 FL	EBA181 FL	Rh5 FL
EIMA001	Accra	5	F	21144.0	0.11	0.00	0.00	0.08	0.00	0.24	0.11	0.03	0.12
EIMA002	Accra	3	M	37259.0	0.06	0.06	0.00	0.05	0.18	0.05	0.09	0.02	0.04
EIMA003	Accra	3	M	38350.0	0.10	0.08	0.00	0.13	0.00	0.08	0.14	0.02	0.02
EIMA004	Accra	9	M		0.56	0.91	0.03	1.12	0.11	2.51	2.67	1.23	0.08
EIMA005	Accra	4	M	31608.0	0.39	0.68	0.16	0.16	0.00	1.04	0.94	0.49	0.29
EIMA012	Accra				0.13	0.08	0.00	0.01	0.00	0.05	0.10	0.12	0.04
EIMA013	Accra	2	M	68637.0	0.19	0.09	0.00	0.00	0.01	0.13	0.12	0.08	0.04
EIMA014	Accra	2	M	1132.0	0.17	0.43	0.00	0.16	0.18	0.16	0.59	0.04	0.14
EIMA015	Accra	3	F	7184.0	0.08	0.06	0.00	0.03	0.16	0.04	0.08	0.02	0.04
EIMA016	Accra	2	M	32612.0	0.05	0.13	0.00	0.03	0.20	0.03	0.06	0.03	0.02
EIMA022	Accra	6	M	5876.0	0.83	0.85	0.61	0.26	0.05	2.77	2.82	1.97	0.21
EIMA023	Accra	10	F	761.0	0.16	0.00	0.00	0.29	0.00	0.28	0.31	0.40	0.08
EIMA024	Accra	10	F	949.0	1.22	1.36	1.11	0.40	0.00	2.22	2.36	1.06	0.09
EIMA026	Accra	4	M	2488.0	0.50	0.47	0.01	0.18	0.04	0.28	0.84	0.10	0.03
EIMA027	Accra	11	M	5580.0	0.16	0.16	0.00	0.00	0.06	0.05	0.10	0.03	0.05
EIMA028	Accra	8	M	18577.0	0.30	0.25	0.00	0.06	0.00	0.11	0.17	0.06	0.07
EIMA029	Accra	2	F	46736.0	0.32	0.24	0.00	0.00	0.00	0.15	0.09	0.04	0.04
EIMA031	Accra	7	F	3889.0	0.06	0.14	0.07	0.26	0.15	0.21	0.04	0.25	0.09
EIMA032	Accra	5	F	44097.0	0.35	0.00	0.00	0.13	0.10	0.08	0.09	0.03	0.07
EIMA033	Accra	9	M	11088.0	0.53	0.92	0.98	0.81	0.00	0.45	1.98	2.01	0.07
EIMA034	Accra	7	M	7399.0	0.03	0.04	0.00	0.02	0.00	0.03	0.00	0.03	0.05

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EIMA046	Accra	12	M	37584.0	0.07	0.02	0.34	0.15	0.00	0.28	0.06	0.56	0.06
EIMA047	Accra	5	M	14288.0	0.26	0.01	0.00	0.00	0.00	0.03	0.03	0.01	0.01
EIMA050	Accra	7	M	8920.0	0.11	0.07	0.12	0.29	0.00	0.35	0.26	0.20	0.09
EIMA052	Accra			893.0	0.13	0.22	0.13	0.18	0.15	0.93	0.70	0.30	0.09
EIMA054	Accra	9	M	26291.0	0.58	0.42	0.08	0.25	0.00	1.42	0.99	0.27	0.03
EIMA055	Accra	2	M	18912.0	0.02	0.03	0.00	0.05	0.01	0.07	0.01	0.07	0.03
EIMA056	Accra	7	M	66629.0	0.30	0.43	0.18	0.27	0.08	0.63	0.56	0.51	0.21
EIMA057	Accra	6	M	117.0	0.13	0.89	0.21	1.07	0.00	2.84	2.76	1.94	2.48
EIMA058	Accra	5	F	26318.0	0.11	0.03	0.00	0.05	0.00	0.36	0.02	0.03	0.04
EIMA059	Accra	11	M	2851.0	0.10	0.05	0.00	0.05	0.18	0.20	0.13	0.03	0.02
EIMA060	Accra	7	M	95531.0	0.06	0.22	0.00	0.15	0.00	0.28	0.43	0.04	0.07
EIMA061	Accra	11	F	115473.0	0.04	0.05	0.00	0.01	0.08	0.05	0.27	0.03	0.08
EIMA062	Accra	5	M	86000.0	0.11	0.05	0.01	0.18	0.00	0.28	0.17	0.04	0.03
EIMA063	Accra	10	F	28528.0	0.06	0.08	0.00	0.03	0.00	0.03	0.00	0.03	0.02
EIMA065	Accra	9	F	715.0	0.41	0.12	0.05	0.09	0.07	0.18	0.32	0.05	0.09
EIMA066	Accra	3	M	80317.0	0.03	0.04	0.00	0.01	0.17	0.02	0.07	0.01	0.01
EIMA067	Accra	6	M	54405.0	0.41	0.06	0.00	0.07	0.00	0.21	0.11	0.04	0.05
EIMA068	Accra	6	M	15932.0	0.42	0.20	0.00	0.10	0.00	0.29	0.21	0.04	0.07
EIMA069	Accra	10	M	20736.0	0.17	1.75	0.39	1.04	0.31	2.16	2.37	0.43	0.11
EIMA070	Accra	8	F	415.0	0.17	0.09	0.01	0.00	0.14	0.28	0.18	0.04	0.03
EIMA071	Accra	3	M	7135.0	0.06	0.07	0.04	0.07	0.00	0.04	0.00	0.04	0.02
EIMA072	Accra	4	F	47518.0	0.40	0.21	0.00	0.03	0.00	0.09	0.08	0.03	0.03
EIMA073	Accra	6	F	8523.0	0.14	0.14	0.00	0.01	0.04	0.07	0.10	0.05	0.07
EIMA074	Accra	2	F	120826.0	0.27	0.55	0.00	0.10	0.13	0.84	1.40	0.05	0.04
EIMA075	Accra	4	F	97309.0	0.35	0.01	0.00	0.08	0.08	0.03	0.06	0.02	0.02
EIMA076	Accra	7	F	84464.0	0.30	0.71	0.66	1.02	0.00	2.62	2.57	1.07	0.07
EIMA077	Accra	4	M	60701.0	0.10	0.09	0.00	0.02	0.12	0.03	0.03	0.01	0.01
EIMA078	Accra	4	M	58485.0	0.26	0.00	0.00	0.00	0.00	0.14	0.07	0.20	0.04
EIMA080	Accra	10	F	123611.0	0.04	0.00	0.00	0.04	0.00	0.01	0.03	0.02	0.01
EIMA083	Accra	2	F	74263.0	0.00	0.11	0.00	0.00	0.00	0.08	0.09	0.03	0.03

EIMA084	Accra	2	F	63597.0	0.00	0.13	0.00	0.03	0.00	0.03	0.02	0.02	0.00
EIMA086	Accra	6	M	75.0	1.14	0.33	0.00	0.94	0.00	2.09	0.77	0.80	0.12
EIMA087	Accra	3	M	102595.0	0.00	0.00	0.00	0.43	0.00	1.47	1.28	0.04	0.02
EIMA088	Accra	3	F	10339.0	0.00	0.05	0.00	0.04	0.00	0.03	0.00	0.04	0.02
EIMA089	Accra	14	F	34109.0	0.93	2.15	0.00	0.09	0.07	2.59	2.80	0.07	0.06
EIMA090	Accra	6	M	11229.0	0.00	0.13	0.00	0.01	0.00	0.02	0.00	0.03	0.01
EIMA091	Accra	9	F	53435.0	0.11	0.06	0.00	0.05	0.00	0.03	0.08	0.02	0.01
EIMA092	Accra	7	F	133556.0	0.32	0.29	0.02	0.79	1.22	1.22	1.56	0.06	0.05
EIMA093	Accra	12	F	14941.0	0.03	0.00	0.00	0.00	0.00	0.07	0.07	0.05	0.07
EIMA094	Accra	2	M	28794.0	0.00	0.00	0.00	0.00	0.00	0.11	0.09	0.01	0.05
EIMA095	Accra	6	M	53928.0	0.00	0.00	0.00	0.00	0.00	0.07	0.05	0.02	0.03
EIMA096	Accra	9	F	6324.0	0.00	0.00	0.00	0.00	0.00	0.03	0.02	0.01	0.03
EIMA097	Accra	6	M	93.0	0.04	0.00	0.00	0.00	0.00	0.02	0.02	0.01	0.02
EIMA098	Accra	5	F	42922.0	0.17	0.04	0.54	0.36	0.29	0.51	0.26	0.91	0.05
EIMA099	Accra	6	M	185767.0	0.28	0.18	0.01	0.45	0.00	0.61	0.78	0.03	0.03
EIMA103	Accra	4	F	82462.0	0.07	0.01	0.00	0.00	0.00	0.05	0.05	0.03	0.05
EIMA105	Accra	4	F	92890.0	0.17	0.00	0.00	0.00	0.41	0.11	0.07	0.01	0.04
EIMA106	Accra	5	F	5471.0	0.08	0.00	0.00	0.00	0.20	0.21	0.11	0.12	0.11
EIMA107	Accra	4	M	15965.0	0.05	0.00	0.00	0.00	0.00	0.02	0.02	0.01	0.03
EIMA108	Accra	11	M	7503.0	0.07	0.00	0.00	0.00	0.10	0.02	0.01	0.01	0.02
EIMA109	Accra	4	M	3495.0	0.10	0.00	0.46	0.11	0.00	0.32	0.16	0.47	0.03
EIMA113	Accra	6	M	4271.0	0.03	0.00	0.00	0.00	0.00	0.03	0.02	0.01	0.01
EIMA116	Accra	3	M	119900.0	0.71	0.08	0.07	0.04	0.04	0.31	0.14	0.01	0.03
EIMA117	Accra	3	F	119.0	0.45	0.56	0.02	0.00	1.01	0.03	0.01	0.00	0.01
EIMA119	Accra	6	F	30483.0	0.73	1.08	0.04	0.00	0.95	0.69	1.62	0.07	0.07
EIMA122	Accra	3	M	41076.0	0.00	0.00	0.00	0.00	0.00	0.03	0.03	0.01	0.02
EIMA123	Accra	3	M	70843.0	0.13	0.03	0.01	0.15	0.09	0.72	0.04	0.01	0.04
EIMA124	Accra	12	F	33838.0	0.10	0.00	0.00	0.00	0.00	0.09	0.05	0.01	0.03
EIMA125	Accra	7	M	98847.0	0.11	0.00	0.00	0.00	0.00	0.01	0.01	0.00	0.01
EIMA126	Accra	4	M	39441.0	0.16	0.00	0.00	0.00	0.00	0.07	0.05	0.00	0.02

EIMA127	Accra	14	M	36613.0	0.43	1.16	0.78	0.21	0.45	0.23	0.68	0.85	0.07
EIMA128	Accra	12	F	75382.0	0.59	1.88	1.39	0.52	0.59	2.50	2.59	1.34	0.03
EIMA129	Accra	3	M	14472.0	0.60	0.32	0.80	0.04	0.00	1.42	0.86	0.68	0.09
EIMA130	Accra	3	M	86.0	0.21	0.00	0.00	0.00	0.00	0.04	0.02	0.01	0.02
EIMA131	Accra	13	F	2763.0	0.19	0.35	0.63	0.53	0.23	0.31	0.43	1.05	0.17
EIMA132	Accra	10	F	100339.0	0.04	0.02	0.00	0.01	0.00	0.04	0.05	0.02	0.03
EIMA133	Accra	8	F	40924.0	0.32	0.06	0.00	0.02	0.01	0.03	0.04	0.01	0.02
EIMA134	Accra	8	F	1584.0	0.20	0.45	0.20	0.03	0.72	0.04	0.03	0.03	0.03
EIMA135	Accra	7	F	9392.0	0.37	0.97	0.43	0.22	0.75	1.09	1.16	0.63	0.09
EIMA136	Accra	5	F	84560.0	0.17	0.47	0.00	0.20	0.00	0.67	2.46	0.09	0.02
EIMA137	Accra			106762.0	0.00	0.08	0.00	0.00	0.00	0.07	0.24	0.03	0.06
EIMA138	Accra	8	M	64101.0	0.00	0.00	0.00	0.11	0.00	0.08	0.10	0.02	0.03
EIMA139	Accra	7	M	14375.0	0.51	0.22	0.11	0.01	0.01	1.82	1.41	1.23	0.97
EIMA140	Accra	9	F	2219.0	0.09	0.12	0.00	0.00	0.00	0.03	0.04	0.02	0.03
EIMA141	Accra	8	M	191.0	0.05	0.00	0.00	0.00	0.00	0.22	0.10	0.02	0.04
EIMA144	Accra	2	F	1737.0	0.00	0.00	0.00	0.00	0.00	0.01	0.01	0.00	0.01
EIMA145	Accra	8	F	340.0	0.15	0.00	0.00	0.00	0.01	1.09	0.13	0.29	0.10
EIMA148	Accra	10	F	7275.0	0.27	0.00	0.00	0.00	0.00	0.21	0.08	0.01	0.03
EIMA149	Accra	6	M	21097.0	0.00	0.00	0.00	0.00	0.00	0.02	0.02	0.01	0.01
EIMA151	Accra	12	M	23923.0	0.23	0.08	0.08	0.00	0.09	0.04	0.05	0.02	0.03
EIMA152	Accra			na	0.14	0.09	0.08	0.08	0.09	0.05	0.04	0.01	0.02
EIMA153	Accra	8	M	43248.0	0.44	0.10	0.10	0.03	0.01	0.02	0.02	0.01	0.02
EIMA154	Accra			na	0.53	0.14	0.12	0.08	0.40	0.05	0.03	0.02	0.02
EIMA155	Accra			na	0.72	0.10	0.12	0.06	0.34	0.09	0.04	0.02	0.03
EIMA156	Accra			105795.0	0.32	0.02	0.00	0.05	0.19	0.12	0.03	0.02	0.02
EIMA157	Accra			10985.0	0.59	0.44	1.03	0.02	1.03	1.55	2.13	0.95	0.04
EIMA158	Accra			44118.0	0.95	0.60	0.11	0.00	1.20	0.55	0.78	0.06	0.06
EIMA159	Accra			32491.0	0.00	0.00	0.00	0.00	0.00	0.03	0.04	0.02	0.02
EIMA160	Accra	3	M	117623.0	0.04	0.18	0.00	0.00	0.00	0.13	0.18	0.02	0.04
EIMA161	Accra	12	F	37733.0	0.50	0.00	0.00	0.00	0.00	0.64	0.26	0.02	0.04

ID	Location	Age	Sex	Value									
EIMA162	Accra			71541.0	0.18	0.00	0.00	0.17	1.05	0.05	0.04	0.03	0.03
EIMA164	Accra			2895.0	0.32	0.00	0.00	0.46	1.05	0.21	0.13	0.08	0.12
EIMA165	Accra			1282.0	0.16	0.09	0.04	0.00	0.45	0.20	0.09	0.02	0.03
EIMA167	Accra			218326.0	0.18	0.05	0.02	0.00	0.00	0.24	0.11	0.06	0.13
EIMA169	Accra			68908.0	0.06	0.00	0.00	0.00	0.00	0.39	0.26	0.26	0.37
EIMA170	Accra			575.0	0.00	0.00	0.11	0.00	0.00	0.10	0.08	0.09	0.05
EIMA171	Accra			21312.0	0.02	0.06	0.00	0.00	0.00	0.02	0.02	0.01	0.01
EIMA172	Accra	9	F	42208.0	0.00	0.00	0.00	0.00	0.00	0.02	0.01	0.01	0.02
EIMA173	Accra	8	M	16091.0	0.50	0.17	0.03	0.26	0.36	0.04	0.02	0.02	0.01
EIMA174	Accra	2	M	95209.0	0.82	0.32	0.12	0.62	0.39	0.93	0.77	0.18	0.03
EIMA175	Accra	10	M	30308.0	1.35	0.00	0.00	0.00	0.00	0.04	0.02	0.01	0.00
EIMA176	Accra	4	M	108961.0	0.16	0.00	0.00	0.00	0.00	0.12	0.05	0.08	0.02
EIMA177	Accra	5	F	10716.0	0.00	0.00	0.00	0.00	0.00	0.02	0.02	0.01	0.01
EIMA178	Accra	3	M	96312.0	0.00	0.00	0.00	0.00	0.00	0.03	0.03	0.01	0.02
EIMK001	Kintampo	3	M	2717.0	0.09	0.01	0.00	0.00	0.00	0.05	0.05	0.04	0.06
EIMK002	Kintampo	4	M	25291.0	0.17	0.05	0.01	0.00	0.00	0.05	0.03	0.03	0.06
EIMK003	Kintampo	2	M	80.0	0.00	0.12	0.02	0.00	0.00	0.11	0.19	0.04	0.01
EIMK004	Kintampo	7	F	67.0	0.00	0.00	0.00	0.00	0.18	0.14	0.06	0.15	0.02
EIMK005	Kintampo	4	M	3739.0	0.00	0.03	0.77	1.41	0.00	2.62	0.63	2.54	0.08
EIMK006	Kintampo	2	F	0.0	0.00	0.07	0.00	0.00	1.17	0.06	0.05	0.09	0.07
EIMK008	Kintampo	2	M	0.0	0.00	0.16	0.03	0.34	0.17	0.87	0.54	0.78	0.10
EIMK009	Kintampo	2	F	24727.0	0.30	0.03	0.00	0.14	0.03	0.11	0.06	0.06	0.06
EIMK010	Kintampo	8	F	18367.0	0.53	0.66	0.06	1.03	0.20	1.61	2.37	0.49	0.05
EIMK011	Kintampo	2	M	257760.0	0.05	0.00	0.00	0.72	0.00	0.58	0.86	0.14	0.03
EIMK012	Kintampo	6	M	312000.0	0.01	0.00	0.00	0.01	0.00	0.03	0.04	0.03	0.04
EIMK013	Kintampo	6	F	2717.0	0.20	0.06	0.00	0.19	0.00	0.07	0.05	0.04	0.04
EIMK015	Kintampo	2	F	19300.0	0.11	0.05	0.14	0.62	0.14	0.41	0.25	0.09	0.06
EIMK016	Kintampo	M		113475.0	0.21	0.11	0.00	0.12	0.00	0.22	0.78	0.05	0.13
EIMK017	Kintampo	3	F	3204.0	0.06	0.02	0.00	0.00	0.03	0.16	0.24	0.05	0.07
EIMK018	Kintampo	2	F	0.0	0.03	0.01	0.00	0.00	0.00	0.01	0.01	0.02	0.01

EIMK019	Kintampo	6	F	7800.0	0.90	0.65	0.21	0.81	0.12	0.62	1.78	0.24	0.15
EIMK020	Kintampo	6	M	142880.0	0.18	0.18	0.05	0.00	0.00	0.81	0.51	0.10	0.09
EIMK021	Kintampo	14	F	10260.0	0.15	0.07	0.12	0.16	0.03	0.89	0.07	0.50	0.04
EIMK022	Kintampo	10	F		0.34	0.23	0.11	0.19	0.06	0.92	1.29	0.38	0.09
EIMK024	Kintampo	4	M	0.0	0.10	0.08	0.00	0.02	0.03	0.08	0.06	0.03	0.01
EIMK025	Kintampo	5	F	27669.0	0.28	0.14	0.00	0.24	0.00	0.18	0.11	0.04	0.04
EIMK028	Kintampo	2	F	1326.0	0.51	0.11	0.00	0.09	0.07	0.22	0.20	0.03	0.03
EIMK032	Kintampo	11	M	712.0	0.16	1.15	1.17	0.76	0.29	2.21	2.54	2.29	0.10
EIMK041	Kintampo	10	M	2988.0	0.49	0.25	0.12	0.02	0.00	0.51	1.58	0.08	0.07
EIMK042	Kintampo	4	F	0.0	0.07	0.02	0.02	0.16	0.00	0.08	0.06	0.16	0.03
EIMK044	Kintampo	4	F	2150.0	0.56	0.40	0.00	1.38	0.21	0.67	1.64	0.18	0.28
EIMk045	Kintampo	8	F	222.2	0.07	0.00	0.00	0.03	0.00	1.60	0.06	0.06	0.03
EIMK047	Kintampo	11	M	655.5	0.84	0.44	0.31	0.21	0.12	0.72	1.80	0.41	0.18
EIMK048	Kintampo	2	M	93.6	0.01	0.37	0.02	0.80	0.03	0.21	0.09	0.08	0.21
EIMK050	Kintampo	14	M	1080.0	0.09	0.18	0.13	0.16	0.03	0.60	0.53	1.25	0.04
EIMK052	Kintampo	3	M	0.0	0.02	0.00	0.00	0.07	0.00	0.07	0.05	0.02	0.01
EIMK055	Kintampo	5	F	0.0	0.50	0.36	0.15	0.00	0.18	0.19	0.06	0.10	0.09
EIMK063	Kintampo	5	M	1953.0	0.11	0.03	1.04	0.01	0.00	0.96	0.04	1.79	0.02
EIMK066	Kintampo	2	F	585.0	0.25	0.02	0.00	0.73	0.06	0.20	2.39	0.12	0.07
EIMK076	Kintampo	4	F	959.0	0.46	0.12	0.13	0.00	1.35	0.40	0.04	0.04	0.04
EIMK077	Kintampo	2	F	248970.0	0.26	0.06	0.06	0.04	0.00	0.67	0.09	0.05	0.04
EIMK081	Kintampo	2	M	59360.0	0.11	0.02	0.00	0.08	0.00	0.05	0.50	0.02	0.01
EIMK084	Kintampo	5	M	73528.0	0.26	0.00	0.00	0.01	0.02	0.15	0.02	0.04	0.06
EIMK087	Kintampo	4	F	47.0	0.22	0.05	0.00	0.30	0.00	0.02	0.08	0.02	0.03
Eimk089	Kintampo	3	M	8365.0	0.11	0.05	0.00	0.36	0.00	0.14	0.15	0.05	0.02
EIMK091	Kintampo	3	F	810300.0	0.10	0.09	0.00	0.86	0.00	0.07	0.20	0.05	0.04
EIMK095	Kintampo	9	F	92664.0	0.06	0.02	0.00	0.03	0.03	0.24	0.05	0.04	0.02
EIMK097	Kintampo	2	M	96.0	0.47	0.10	0.28	0.04	0.02	0.12	0.06	0.04	0.04
EIMK098	Kintampo	3	F	3013.0	0.16	0.14	0.00	0.00	0.13	2.28	0.04	1.10	0.03
EIMK105	Kintampo	9	F	173250.0	0.13	0.02	0.00	0.00	0.00	0.12	0.05	0.03	0.05

EIMK106	Kintampo	7	F	0.0	0.10	0.15	0.02	0.41	0.00	2.16	0.35	0.05	0.04
EIMK108	Kintampo	5	M	73005.0	0.11	0.06	0.00	0.00	0.00	0.13	0.20	0.03	0.03
EIMK110	Kintampo	14	F	3013.0	0.24	0.39	0.10	0.53	0.00	0.71	0.40	0.11	0.01
EIMK113	Kintampo	5	F	213210.0	0.05	0.03	0.00	0.07	0.00	0.13	0.05	0.03	0.02
EIMK114	Kintampo	5	M	159900.0	0.11	0.02	0.00	0.79	0.00	0.27	2.50	0.10	0.05
EIMK119	Kintampo	8	M	4109.0	0.06	0.04	0.00	0.10	0.00	0.04	0.21	0.02	0.02
EIMK121	Kintampo	3	M	0.0	0.28	1.24	0.34	0.03	0.13	2.10	0.06	1.90	0.14
EIMK122	Kintampo	4	F	454250.0	0.00	0.00	0.00	1.21	0.00	0.05	2.75	0.20	0.02
EIMK124	Kintampo	2	F	595700.0	0.07	0.04	0.00	0.24	0.03	0.27	0.74	0.23	0.10
EIMK125	Kintampo	3	F	384300.0	0.19	0.05	0.00	0.66	0.00	0.20	0.10	0.05	0.03
EIMK131	Kintampo	5	F	110160.0	0.11	0.05	0.00	0.39	0.00	0.03	0.84	0.08	0.02
EIMK132	Kintampo	9	F	38640.0	0.08	0.07	0.00	0.47	0.10	0.28	0.23	0.10	0.02
EIMK133	Kintampo	3	M	53550.0	0.05	0.10	0.00	0.00	0.00	0.28	0.06	0.05	0.04
EIMK134	Kintampo	8	M	29.0	0.49	0.20	0.43	0.11	0.39	0.72	0.07	0.72	0.04
EIMK135	Kintampo	5	M	212660.0	0.15	0.01	0.02	0.00	0.00	0.17	0.03	0.03	0.03
EIMK136	Kintampo	3	M	171000.0	0.09	0.03	0.00	0.62	0.00	0.05	0.34	0.07	0.01
EIMK137	Kintampo	7	F	0.0	0.17	0.00	0.00	0.08	0.00	0.05	0.45	0.05	0.04
EIMK139	Kintampo	8	F	306.0	0.38	0.06	0.04	0.04	0.00	0.49	0.77	0.06	0.04
EIMK140	Kintampo	12	F	299800.0	0.20	0.04	0.08	0.20	0.15	1.21	0.19	0.05	0.06
EIMK141	Kintampo	2	M	196000.0	0.00	0.00	0.00	0.00	0.00	0.03	0.05	0.04	0.01
EIMK144	Kintampo	12	M	16749.0	0.42	0.88	0.20	0.09	0.45	0.88	0.08	0.91	0.09
EIMK145	Kintampo	11	M	8.0	0.23	0.06	0.00	0.00	0.00	0.20	0.10	0.08	0.06
EIMK146	Kintampo	6	F	0.0	0.10	0.36	0.80	0.22	0.08	1.41	0.48	2.15	0.10
EIMK147	Kintampo	8	M	328860.0	0.07	0.01	0.00	0.00	0.00	0.09	0.09	0.16	0.09
EIMK148	Kintampo	5	F	0.0	0.01	0.04	0.05	0.00	0.08	0.05	0.18	0.12	0.03
EIMK149	Kintampo	10	F	7038.0	0.12	1.68	1.65	0.34	0.01	2.85	0.07	2.49	0.09
EIMK150	Kintampo	7	M	39804.0	0.41	0.01	0.31	0.07	0.00	0.75	1.20	0.84	0.03
EIMK152	Kintampo	14	M	19244.0	0.36	0.14	0.00	0.01	0.55	0.73	0.21	0.36	0.07
EIMK155	Kintampo	12	M	23813.0	0.05	0.25	0.92	0.00	0.16	1.01	0.06	1.03	0.03
EIMK156	Kintampo	3	F	33872.0	0.02	0.01	0.00	0.01	0.14	0.34	0.05	1.35	0.05

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EIMK157	Kintampo	4	F	36600.0	0.00	0.00	0.00	0.65	0.00	0.33	0.23	0.09	0.06
EIMK158	Kintampo	9	F	0.0	0.17	0.23	0.02	0.03	0.04	0.86	0.09	0.04	0.04
EIMK159	Kintampo	7	F	33872.0	0.00	0.09	0.04	0.15	0.02	0.30	0.19	0.24	0.02
EIMK160	Kintampo	12	F	36600.0	0.27	0.57	0.28	0.10	0.17	0.53	0.12	0.95	0.18
EIMK161	Kintampo	6	F	3100.0	0.34	0.16	0.00	0.00	0.10	0.76	0.40	0.14	0.09
EIMK163	Kintampo	8	F	38836.0	0.00	1.32	0.06	0.74	0.04	0.51	2.66	0.04	0.02
EIMK164	Kintampo	7	M	3154.0	0.00	0.35	0.07	0.21	0.21	0.32	0.05	0.21	0.38
EIMK165	Kintampo	3	F	26398.0	0.00	0.00	0.00	0.19	0.00	0.11	0.27	0.14	0.03
EIMK168	Kintampo	5	M	19809.0	0.00	0.00	0.00	0.49	0.00	0.78	0.07	0.29	0.03
EIMK169	Kintampo	6	M	3154.0	0.21	0.16	0.00	0.06	0.35	0.34	0.80	0.24	0.07
EIMK170	Kintampo	3	M	5170.0	0.00	0.26	0.03	0.07	0.08	1.24	0.02	0.43	0.52
EIMK173	Kintampo	9	M	211460.0	0.07	0.11	0.02	0.29	0.00	0.07	0.11	0.05	0.37
EIMK174	Kintampo	2	M	479940.0	0.00	0.28	0.18	0.02	0.00	0.18	0.12	0.05	0.06
EIMK176	Kintampo	6	F	124745.0	0.04	0.15	0.01	0.11	0.00	0.45	0.03	0.05	0.04
EIMK178	Kintampo	4	F	0.0	0.03	0.07	0.35	0.07	0.00	0.91	0.12	0.60	0.03
EIMK179	Kintampo	5	F	1234640.0	0.08	0.03	0.03	1.27	0.00	0.63	2.71	0.35	0.04
EIMK200	Kintampo	4	M	12694.0	0.19	0.18	0.04	0.00	0.00	0.08	0.03	0.11	0.07
EIMK201	Kintampo	14	M	72709.0	1.26	0.10	0.15	0.29	0.00	0.15	0.10	0.06	0.03
EIMK204	Kintampo	14	M	458.0	0.24	0.72	0.02	0.00	0.69	0.88	0.01	0.41	0.08
EIMK209	Kintampo	4	F	475860.0	0.36	0.09	0.05	0.08	0.02	0.09	0.16	0.04	0.03
EIMK210	Kintampo	6	M	227205.0	0.08	0.05	0.03	0.00	0.00	0.20	0.07	0.03	0.01
EIMK211	Kintampo	2	M	141375.0	0.19	0.15	0.13	0.08	0.00	0.16	0.16	0.16	0.04
EIMK219	Kintampo	2	M	240465.0	0.47	0.33	0.06	0.02	0.00	0.66	0.04	0.05	0.03
EIMK220	Kintampo	7	M	12275.0	0.90	1.70	0.38	0.00	0.22	2.12	0.02	0.59	0.19
EIMK221	Kintampo	6	M	520520.0	0.26	0.06	0.02	0.27	0.00	0.08	0.43	0.02	0.02
EIMK227	Kintampo	11	M	218400.0	0.20	0.53	0.09	0.33	0.00	0.09	0.10	0.11	0.04
EIMK231	Kintampo	6	M	238580.0	0.10	0.08	0.04	0.36	0.00	0.15	0.28	0.17	0.03
EIMK235	Kintampo	3	M	18414.0	0.04	0.06	0.02	0.17	0.00	1.35	0.50	0.04	0.03
EIMK240	Kintampo	13	M	95890.0	0.10	0.07	0.04	1.52	0.00	0.02	2.74	0.02	0.02
EIMK241	Kintampo	3	M	280000.0	0.50	0.00	0.00	0.00	0.00	0.23	0.06	0.06	0.03

EIMK242	Kintampo	7	M	373230.0	0.14	0.10	0.22	0.00	0.00	0.26	0.04	0.79	0.09
EIMK244	Kintampo	2	M	820600.0	0.09	0.01	0.00	0.81	0.00	0.06	0.07	0.02	0.02
EIMK246	Kintampo	2	M	224660.0	0.16	0.32	0.02	0.09	0.00	0.03	0.17	0.01	0.01
EIMK247	Kintampo	12	M	9800.0	0.95	1.23	1.75	0.00	0.54	2.45	1.14	2.72	1.01
EIMK248	Kintampo	9	M	98400.0	0.05	0.65	0.02	0.00	0.00	0.23	0.02	0.08	0.01
EIMK249	Kintampo	9	M	77715.0	0.10	0.18	0.03	0.31	0.00	0.26	0.43	0.21	0.01
EIMK251	Kintampo	4	M	115110.0	0.20	0.25	0.04	0.03	0.00	0.01	0.04	0.01	0.02
EIMK252	Kintampo	5	M	191820.0	0.07	0.10	0.00	0.00	0.00	0.04	0.02	0.04	0.01
EIMK253	Kintampo	7	F	84280.0	0.08	0.16	0.00	0.00	0.00	0.53	0.14	0.06	0.06
EIMK255	Kintampo	8	M	37310.0	0.02	0.18	0.00	0.32	0.00	0.21	0.23	0.08	0.03
EIMK258	Kintampo	11	M	112860.0	0.16	0.10	0.20	0.00	0.09	0.11	0.01	0.72	0.03
EIMK265	Kintampo	2	M	752100.0	0.31	0.02	0.01	0.01	0.00	0.03	0.10	0.02	0.02
EIMK266	Kintampo	5	F	14107.0	0.08	0.05	0.21	0.11	0.00	0.38	0.04	0.90	0.02
EIMK267	Kintampo	3	M	490230.0	0.14	0.39	0.15	0.22	0.00	0.14	0.63	0.03	0.06
EIMK268	Kintampo	10	M	225525.0	0.12	0.06	0.02	0.21	0.00	0.36	0.09	0.16	0.01
EIMK274	Kintampo	3	F	184470.0	0.78	0.25	0.02	0.00	0.00	0.71	0.04	0.04	0.04
EIMK275	Kintampo	6	F	1207.0	1.63	1.50	1.93	0.03	0.29	2.92	0.15	2.21	0.06
EIMK280	Kintampo	14	F	6079.0	0.07	0.03	0.00	0.01	0.07	0.15	0.06	0.05	0.04
EIMK281	Kintampo	3	F	1040.0	0.26	0.07	0.12	0.16	0.00	0.19	0.06	0.21	0.05
EIMK284	Kintampo	6	M	111230.0	0.05	0.05	0.28	0.21	0.00	0.17	2.15	0.45	0.03
EIMK286	Kintampo	2	F	251640.0	0.11	0.15	0.08	0.10	0.00	0.56	1.03	0.02	0.03
EIMK288	Kintampo	5	F	129220.0	0.88	0.11	1.25	0.06	0.00	2.44	0.21	1.47	0.03
EIMK293	Kintampo	2	F	127395.0	0.04	0.01	0.00	0.91	0.00	0.03	1.67	0.02	0.04
EIMK296	Kintampo	5	F	5000.0	0.19	0.06	0.03	0.00	0.00	0.31	1.14	0.02	0.03
EIMK297	Kintampo	3	M	216815.0	0.17	0.12	0.06	0.09	0.00	0.12	0.13	0.34	0.02
EIMK300	Kintampo	4	F	86840.0	0.00	0.05	0.00	0.26	0.00	0.14	0.62	0.03	0.03
EIMK301	Kintampo	2	M	106745.0	0.42	0.02	0.00	0.14	0.00	0.47	0.33	0.04	0.03
EIMK303	Kintampo	4	M	88425.0	0.04	0.08	0.33	0.00	0.00	0.34	0.04	1.45	0.02
EIMK304	Kintampo	8	F	280830.0	0.04	0.00	0.00	0.04	0.00	0.08	0.08	0.01	0.01
EIMK306	Kintampo	7	M	223095.0	0.13	0.02	0.01	0.08	0.00	0.44	0.06	0.23	0.07

EIMK307	Kintampo	4	M	67425.0	0.06	0.02	0.00	0.04	0.00	0.06	0.09	0.22	0.06
EIMK309	Kintampo	7	F	302940.0	0.36	0.10	0.39	0.07	0.00	1.31	0.12	0.92	0.11
EIMK312	Kintampo	2	M	362700.0	0.08	0.27	0.00	0.55	0.00	0.11	0.38	0.02	0.02
EIMK313	Kintampo	4	M	12040.0	0.12	0.07	0.51	0.03	0.00	0.17	0.04	1.86	0.03
EIMK314	Kintampo	3	M	208.0	0.00	0.22	0.34	0.31	0.00	0.54	1.06	1.10	0.04
EIMK315	Kintampo	2	F	153850.0	0.21	0.04	1.32	0.04	0.00	0.93	0.08	1.43	0.07
EIMK317	Kintampo	6	F	11748.0	0.16	0.10	0.01	0.54	0.00	0.09	0.08	0.17	0.03
EIMK318	Kintampo	5	M		0.23	1.42	1.00	0.03	0.00	1.10	0.06	1.88	0.02
EIMK319	Kintampo	5	M		0.17	0.55	0.05	0.00	0.00	0.41	0.06	0.10	0.09
EIMK320	Kintampo	3	F	375385.0	0.05	0.06	0.24	0.00	0.00	0.05	1.75	0.83	0.03
EIMK321	Kintampo	5	M	100.0	0.41	0.10	0.08	0.12	0.05	1.97	1.14	1.53	0.10
EIMK328	Kintampo	5	F	148980.0	0.00	0.30	0.42	0.05	0.00	0.15	0.18	0.30	0.04
EIMK329	Kintampo	11	M	4096.0	0.24	0.17	0.04	0.00	0.00	0.10	0.08	0.43	0.08
EIMK331	Kintampo	7	M	434196.0	0.12	0.02	0.35	0.26	0.00	0.87	0.16	1.37	0.03
EIMK335	Kintampo	5	F	500940.0	0.06	0.05	0.06	0.10	0.00	0.07	0.23	0.31	0.04
EIMK338	Kintampo	2	M	686400.0	0.05	0.00	0.00	0.10	0.00	0.09	1.26	0.01	0.03
EIMK339	Kintampo	5	M	742500.0	0.18	0.15	0.00	0.00	0.00	0.06	1.50	0.11	0.05
EIMK341	Kintampo	4	F	101495.0	0.09	0.06	0.01	0.31	0.00	0.34	0.32	0.38	0.08
EIMK342	Kintampo	7	M	197880.0	0.09	0.10	0.11	0.00	0.00	0.14	0.15	0.36	0.01
EIMK351	Kintampo	4	F	30843.0	0.24	0.13	0.00	0.59	0.00	0.83	1.80	0.06	0.05
EIMK353	Kintampo	5	M	17275.0	1.58	0.49	1.45	0.00	0.00	1.57	0.04	1.61	0.05
EIMK359	Kintampo	3	F	40141.0	0.07	0.11	0.01	0.33	0.00	0.04	0.16	0.02	0.03
EIMK365	Kintampo	5	M	114660.0	0.20	0.31	0.36	0.14	0.00	0.61	0.24	0.53	0.04
EIMK370	Kintampo	8	F	112860.0	0.22	0.27	0.00	0.15	0.00	0.13	0.11	0.03	0.04
EIMK374	Kintampo	7	M	141750.0	0.12	0.11	0.20	0.16	0.00	1.13	0.06	0.25	0.03
EIMK375	Kintampo	2	F	82800.0	0.04	0.13	0.02	0.09	0.08	0.06	0.55	0.02	0.02
EIMK376	Kintampo	2	F	1485800.0	0.00	0.01	0.00	0.04	0.04	0.21	0.04	0.07	0.05
EIMK377	Kintampo	4	F	88350.0	0.11	0.12	0.14	0.00	0.00	0.74	0.05	0.67	0.03
EIMK380	Kintampo	6	F	10556.0	0.15	0.29	0.24	0.00	0.00	0.65	0.03	0.90	0.06
EIMK384	Kintampo	13	F	180600.0	0.06	0.08	0.06	0.06	0.00	0.25	0.06	0.16	0.05

EIMK388	Kintampo	3	M	41108.0	0.46	0.00	0.00	0.00	0.00	0.07	0.08	0.10	0.08
EIMN005	Navrongo	8	M	37410.0	0.16	0.14	0.08	0.25	0.00	0.78	2.00	0.39	0.24
EIMN008	Navrongo	3	M	44203.5	0.16	0.09	0.00	0.00	0.00	0.08	0.06	0.02	0.03
EIMN009	Navrongo	2	M	23843.5	0.10	0.13	0.11	0.02	0.00	0.10	0.20	0.05	0.02
EIMN011	Navrongo	7	M	40509.0	0.34	0.13	0.01	0.00	0.00	2.11	0.63	0.67	0.05
EIMN013	Navrongo	12	F	38709.0	0.21	0.32	0.13	0.54	0.00	1.66	0.50	1.71	0.28
EIMN014	Navrongo				0.30	0.03	0.00	0.00	0.00	0.09	0.05	0.06	0.04
EIMN017	Navrongo	3	F	48894.0	0.02	0.04	0.00	0.00	0.00	0.05	0.06	0.03	0.03
EIMN018	Navrongo	2	F	46992.0	0.12	0.03	0.00	0.00	0.00	0.10	0.11	0.02	0.02
EIMN020	Navrongo	4	F	26567.0	0.03	0.08	0.01	0.00	0.00	0.07	0.06	0.03	0.02
EIMN022	Navrongo	9	M	42511.5	0.06	0.04	0.00	0.27	0.26	0.45	0.28	0.12	0.04
EIMN027	Navrongo	2	M	78986.0	0.14	0.05	0.00	0.01	0.00	0.43	0.12	0.05	0.02
EIMN029	Navrongo	3	M	39032.0	0.16	0.03	0.02	0.14	0.03	0.42	0.12	0.05	0.01
EIMN031	Navrongo	5	F	83818.5	0.07	0.15	0.08	0.03	0.00	0.03	0.06	0.02	0.02
EIMN032	Navrongo	5	F	20775.5	0.00	0.00	0.01	0.00	0.00	0.04	0.07	0.10	0.01
EIMN035	Navrongo				0.52	0.14	0.15	0.03	0.03	0.62	0.51	0.11	0.05
EIMN036	Navrongo	2	F	20210.0	0.38	0.03	0.08	0.11	0.04	0.10	0.04	0.03	0.03
EIMN039	Navrongo	7	M	108530.5	0.11	0.07	0.05	0.07	0.00	0.10	0.40	0.37	0.04
EIMN041	Navrongo	9	F	23435.0	0.19	0.09	0.50	0.26	0.06	0.33	0.20	1.36	0.05
EIMN042	Navrongo	6	F	112464.0	0.60	0.04	0.00	0.03	0.22	0.29	0.30	0.27	0.30
EIMN043	Navrongo	11	M	49086.0	0.33	0.44	0.22	0.58	0.00	0.52	0.31	0.07	0.07
EIMN044	Navrongo				0.35	0.18	0.24	0.12	0.14	1.02	0.24	0.26	0.03
EIMN045	Navrongo				0.16	0.09	0.34	0.39	0.00	1.66	0.88	0.40	0.03
EIMN047	Navrongo				0.07	0.02	0.01	0.45	0.00	0.43	0.18	1.34	0.03
EIMN051	Navrongo	8	F	54112.5	0.05	0.03	0.02	0.17	0.00	0.02	0.01	0.01	0.02
EIMN052	Navrongo	8	F	13405.5	0.26	0.07	0.03	0.00	0.00	0.05	0.08	0.03	0.04
EIMN053	Navrongo	2	M	12834.0	0.05	0.04	0.02	0.03	0.00	0.01	0.01	0.01	0.01
EIMN054	Navrongo	10	M	38269.0	0.04	0.01	0.02	0.17	0.00	0.82	0.04	0.43	0.03
EIMN056	Navrongo	2	M	9373.0	0.14	0.12	0.03	0.08	0.04	0.24	0.43	0.04	0.04
EIMN057	Navrongo	2	F	403918.0	0.23	0.03	0.03	0.05	0.00	0.03	0.06	0.03	0.05

EIMN060	Navrongo	3	M	16608.0	0.09	0.14	0.03	0.14	0.09	0.36	0.24	0.09	0.09
EIMN062	Navrongo	3	F	95370.0	0.83	0.21	0.17	0.18	0.21	0.64	0.29	0.28	0.04
EIMN063	Navrongo	2	F	108931.0	0.00	0.19	0.03	0.10	0.11	0.28	0.24	0.08	0.03
EIMN064	Navrongo	2	M	60588.0	0.74	0.18	0.03	0.24	0.00	0.29	0.08	0.22	0.02
EIMN066	Navrongo	3	M	13924.0	0.12	0.05	0.07	0.01	0.00	0.05	0.03	0.01	0.02
EIMN070	Navrongo	2	M	40950.0	0.16	0.15	0.02	0.27	0.05	0.54	0.16	0.04	0.05
EIMN073	Navrongo	7	F	42055.0	0.03	0.66	0.17	0.22	0.04	0.89	1.18	0.48	0.03
EIMN079	Navrongo	4	F	16737.5	0.03	0.01	0.01	0.01	0.00	0.05	0.06	0.01	0.02
EIMN083	Navrongo	2	M	35022.0	0.09	0.05	0.04	0.07	0.03	0.22	0.03	0.11	0.04
EIMN084	Navrongo	6	M	54900.0	0.00	0.02	0.01	0.18	0.00	0.29	0.09	0.33	0.05
EIMN085	Navrongo	2	M	74504.0	0.26	0.07	0.05	0.07	0.02	0.07	0.19	0.05	0.04
EIMN086	Navrongo	6	F	89625.0	0.07	0.03	0.02	0.03	0.00	0.07	0.07	0.05	0.06
EIMN087	Navrongo	7	M	2430.0	0.10	0.08	0.11	0.18	0.01	0.31	0.09	0.22	0.03
EIMN088	Navrongo	5	M	7400.0	0.04	0.03	0.04	0.19	0.06	0.63	0.30	0.04	0.02
EIMN093	Navrongo	5	F	157777.5	0.10	0.04	0.09	0.02	0.00	1.20	0.04	0.34	0.04
EIMN096	Navrongo	5	F	14490.0	0.02	0.00	0.04	0.08	0.01	0.07	0.04	0.35	0.04
EIMN097	Navrongo	6	F	16540.0	0.12	0.07	0.12	0.11	0.07	0.05	0.04	0.32	0.06
EIMN099	Navrongo	4	M	24990.0	0.00	0.00	0.00	0.00	0.00	0.03	0.03	0.03	0.02
EIMN100	Navrongo	4	F	36936.0	0.30	0.10	0.33	1.19	0.01	1.43	0.09	1.58	0.02
EIMN103	Navrongo	13	M	38931.0	0.21	0.07	0.32	0.10	0.00	0.36	0.80	0.71	0.02
EIMN104	Navrongo	8	M	66611.5	0.20	0.11	0.47	0.04	0.01	0.42	0.76	1.15	0.17
EIMN105	Navrongo	5	M	75888.0	0.12	0.05	0.10	0.19	0.01	0.09	0.09	0.05	0.04
EIMN106	Navrongo	5	M	71706.0	0.17	0.00	0.02	0.09	0.00	0.07	0.07	0.12	0.11
EIMN108	Navrongo	5	M	48453.0	0.03	0.01	0.01	0.00	0.00	0.10	0.06	0.51	0.00
EIMN109	Navrongo	2	M	41223.0	0.00	0.00	0.05	0.00	0.00	0.07	0.03	0.05	0.03
EIMN113	Navrongo	3	F	38056.0	0.09	0.00	0.00	0.03	0.09	0.63	0.12	0.09	0.02
EIMN114	Navrongo	2	M	53782.5	0.19	0.10	1.41	0.30	0.11	0.34	0.38	1.86	0.02
EIMN116	Navrongo	2	M	35453.0	0.16	0.01	0.01	0.00	0.00	0.05	0.03	0.02	0.02
EIMN120	Navrongo	2	M	32508.0	0.41	0.40	0.09	0.09	0.14	1.00	0.47	0.18	0.15
EIMN124	Navrongo	2	M	15408.0	1.20	0.03	0.11	0.09	0.00	0.35	0.10	0.11	0.02

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EIMN126	Navrongo				0.10	0.06	0.03	0.37	0.13	0.04	0.04	0.02	0.03
EIMN131	Navrongo	11	F	25466.0	0.00	0.00	0.01	0.00	0.00	0.16	0.07	0.29	0.04
EIMN137	Navrongo				0.00	0.00	0.04	0.00	0.00	0.05	0.05	0.05	0.03
EIMN138	Navrongo	5	M	64701.0	0.23	1.54	0.95	0.65	0.08	1.37	1.95	1.91	0.03
EIMN143	Navrongo	4	F	26418.0	0.46	0.05	0.05	0.33	0.00	1.04	0.20	0.60	0.03
EIMN144	Navrongo	6	F	9517.5	0.15	0.04	0.10	0.08	0.00	0.09	0.04	0.02	0.01
EIMN145	Navrongo	7	M	10125.0	0.62	0.10	0.21	0.08	0.01	0.30	0.75	0.11	0.02
EIMN151	Navrongo	12	F	40050.0	0.07	0.12	0.07	0.54	0.02	0.82	0.22	0.02	0.03
EIMN152	Navrongo	6	F	48302.5	0.19	0.41	0.04	0.18	0.33	1.20	0.39	0.19	0.06
EIMN154	Navrongo	2	M	10114.0	0.33	0.00	0.00	0.00	0.07	0.92	0.78	0.08	0.06
EIMN156	Navrongo	3	M	50058.0	0.24	0.00	0.15	0.00	0.00	0.83	0.24	0.12	0.02
EIMN164	Navrongo	6	M	61054.0	0.08	0.01	1.36	0.83	0.00	1.30	0.37	2.25	0.03
EIMN166	Navrongo	7	F	59303.5	0.16	0.01	0.07	0.19	0.00	0.43	0.15	0.14	0.01
EIMN176	Navrongo	2	M	47987.0	0.07	0.04	0.40	0.11	0.07	0.14	0.05	0.21	0.02
EIMN180	Navrongo	5	M	74784.0	0.28	0.02	0.10	0.09	0.00	0.30	0.29	0.02	0.02
EIMN185	Navrongo	10	F	8970.0	0.32	0.09	0.12	0.81	0.15	1.87	0.45	0.71	0.08
EIMN187	Navrongo	4	M	8226.0	0.01	0.00	0.02	0.00	0.00	0.05	0.02	0.02	0.03
EIMN192	Navrongo	5	M	65281.0	0.12	0.20	0.04	0.13	0.00	0.60	0.77	0.10	0.03
EIMN196	Navrongo	6	M	112811.0	0.16	0.12	0.00	0.13	0.00	0.22	0.05	0.03	0.01
EIMN199	Navrongo	4	F	206000.0	0.83	0.53	1.49	0.60	0.68	2.42	0.85	1.21	0.07
EIMN200	Navrongo	3	F	98040.0	0.06	0.10	1.06	0.66	0.00	0.47	0.16	1.71	0.02
EIMN201	Navrongo	6	M	44440.0	0.20	0.17	0.58	0.43	0.00	0.22	0.20	1.68	0.04
EIMN202	Navrongo	2	F	22000.0	0.21	0.11	0.02	0.00	0.00	0.04	0.03	0.03	0.03
EIMN203	Navrongo	2	M	29606.0	0.07	0.07	0.05	0.04	0.00	0.03	0.02	0.02	0.03
EIMN205	Navrongo	6	M	218902.5	0.16	0.13	0.02	0.28	0.00	0.52	0.17	1.55	0.04
EIMN206	Navrongo	3	F	5250.0	0.19	0.07	0.02	0.00	0.00	0.78	0.51	0.13	0.02
EIMN207	Navrongo	5	F	6552.0	0.13	0.46	0.03	0.11	0.57	1.31	0.34	0.08	0.85
EIMN209	Navrongo	4	M	14364.0	0.27	0.92	0.02	0.11	0.20	0.19	2.64	0.28	0.06
EIMN212	Navrongo	5	F	70933.0	0.06	0.05	0.08	0.00	0.00	0.08	0.05	0.03	0.05
EIMN215	Navrongo	5	M	78706.5	0.05	0.03	0.06	0.11	0.00	0.21	0.73	0.30	0.02

EIMN219	Navrongo				0.19	0.10	0.06	0.04	0.00	0.18	0.14	0.02	0.01
EIMN221	Navrongo	4	F	10010.0	0.15	0.07	0.01	0.21	0.00	0.09	0.39	0.06	0.00
EIMN223	Navrongo	8	F	25499.0	0.00	0.28	0.08	0.00	0.00	0.19	0.11	0.52	0.05
EIMN224	Navrongo	10	F	15745.0	0.00	0.12	0.11	0.29	0.02	0.84	0.11	0.64	0.02
EIMN225	Navrongo	4	F	3108.0	0.25	0.30	0.10	0.60	0.29	1.94	1.15	0.09	0.04
EIMN226	Navrongo	5	F	33246.5	0.01	0.23	0.03	0.08	0.00	0.05	0.12	0.03	0.02
EIMN227	Navrongo	8	F	18123.5	0.00	0.00	0.07	0.20	0.00	0.07	0.08	0.03	0.02
EIMN228	Navrongo	4	F	16933.5	0.17	0.94	1.06	0.22	0.00	2.49	2.18	0.92	0.05
EIMN229	Navrongo	4	M	66872.0	0.00	0.00	0.40	0.00	0.04	0.43	0.11	0.63	0.01
EIMN231	Navrongo	6	M	29083.5	1.01	0.05	0.96	0.32	0.00	0.77	0.60	1.96	0.31
EIMN232	Navrongo	3	F	16632.0	0.71	0.32	0.16	0.51	0.17	1.52	1.02	1.15	0.03
EIMN233	Navrongo	5	F	15410.5	0.34	0.19	0.00	0.54	0.00	1.79	0.21	2.11	0.07
EIMN235	Navrongo	2	F	12996.0	0.12	0.06	0.02	0.05	0.00	0.03	0.08	0.04	0.04
EIMN237	Navrongo	7	M	5985.0	0.25	0.21	0.00	0.51	0.00	0.56	0.22	0.14	0.07
EIMN238	Navrongo				0.21	0.08	0.09	0.72	0.00	1.42	0.13	1.50	0.04
EIMN240	Navrongo	3	M	35952.0	0.31	0.25	0.06	0.27	0.00	0.05	0.07	0.15	0.04
EIMN242	Navrongo	3	F	15137.5	0.44	0.34	0.19	0.05	0.00	0.86	0.40	0.38	0.04
EIMN243	Navrongo	9	F	35188.0	1.14	0.45	0.08	0.77	0.00	1.94	2.42	1.03	0.11
EIMN245	Navrongo	8	M	38115.0	0.13	0.65	0.00	0.09	0.11	0.51	0.32	0.13	0.01
EIMN247	Navrongo	9	F	11700.0	1.37	0.66	0.95	0.09	0.10	0.72	0.28	0.93	0.05
EIMN249	Navrongo	5	F	64714.0	0.11	0.16	0.02	0.23	0.22	0.42	0.12	0.04	0.04
EIMN250	Navrongo	11	M	30388.0	0.10	0.02	0.25	0.22	0.15	0.09	0.16	1.04	0.07
EIMN251	Navrongo	2	F	37422.0	0.17	0.08	0.01	0.05	0.08	0.05	0.23	0.79	0.16
EIMN253	Navrongo	9	F	61290.0	0.05	0.06	0.00	0.09	0.00	0.58	0.44	0.31	0.05
EIMN255	Navrongo	8	F	25812.5	0.21	0.04	0.00	0.05	0.00	0.16	0.01	0.04	0.03
EIMN256	Navrongo				0.45	0.10	0.00	0.05	0.06	0.43	0.28	0.03	0.02
EIMN261	Navrongo	3	F	91162.5	0.08	0.01	0.00	0.01	0.11	0.05	0.02	0.03	0.02
EIMN263	Navrongo	3	F	23296.0	0.07	0.03	0.03	0.06	0.24	0.15	0.21	0.08	0.13
EIMN265	Navrongo	2	F	17370.0	0.17	0.24	0.00	0.16	0.00	0.21	0.21	0.02	0.02
EIMN272	Navrongo	10	F	24648.0	0.48	0.14	0.04	0.07	0.00	0.45	0.15	0.32	0.02

EIMN276	Navrongo	2	F	21070.0	0.39	0.95	0.08	0.35	0.00	1.29	1.33	0.26	0.08
EIMN278	Navrongo	3	F	1617.0	0.19	0.05	0.00	0.09	0.00	0.20	0.05	0.02	0.01
EIMN279	Navrongo	2	M	83058.5	0.12	0.08	0.00	0.28	0.14	0.44	0.23	0.05	0.03
EIMN281	Navrongo	11	M	7938.0	0.30	0.06	0.00	0.31	0.08	0.36	0.08	0.30	0.03
EIMN283	Navrongo	7	F	54926.0	0.03	0.03	0.00	0.10	0.11	0.10	0.11	0.14	0.04
EIMN286	Navrongo				0.58	0.25	0.08	0.41	0.07	0.10	0.12	0.34	0.04
EIMN288	Navrongo	9	F	24475.0	0.54	0.15	0.12	0.47	0.00	0.11	0.10	0.34	0.04
EIMN289	Navrongo	2	F	89904.5	0.05	0.12	0.00	0.02	0.11	0.01	0.08	0.01	0.02
EIMN290	Navrongo				0.15	0.14	0.02	0.03	0.00	0.02	0.00	0.11	0.09
EIMN293	Navrongo	3	F	92225.0	0.18	0.14	0.00	0.11	0.00	0.24	0.07	0.04	0.03
EIMN300	Navrongo	3	M	11235.5	0.04	0.00	0.00	0.15	0.00	0.10	0.36	0.03	0.02
EIMN301	Navrongo	4	M	44784.0	0.13	0.15	0.74	0.45	0.13	1.01	0.47	2.08	0.06

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Table A 2. Antibody levels of semi-immune adults living in Kintampo against EBA and Rh antigens measured as ODs.

Sample	Age (in years)	Sex	Antibody levels (Absorbance450)								
			EBA140	EBA175	EBA181	Rh2.2030	Rh4.2	EBA140 FL	EBA175 FL	EBA181 FL	Rh5 FL
EIMKB001	30	M	0.35	0.09	0.00	0.00	0.00	0.05	0.06	0.05	0.01
EIMKB002	33	M	0.30	0.14	0.00	0.02	0.00	0.32	0.03	0.36	0.01
EIMKB003	30	M	0.23	0.27	0.06	0.01	0.01	0.40	1.19	0.16	0.00
EIMKB004	38	M	0.66	0.36	0.12	0.04	0.51	0.15	0.38	0.05	0.10
EIMKB005	32	M	0.50	0.80	0.47	0.74	0.00	0.30	0.99	0.58	0.09
EIMKB006	31	M	0.46	0.41	0.00	0.03	0.00	0.26	0.65	0.10	0.07
EIMKB007	36	M	0.49	0.27	0.14	0.00	0.00	0.12	0.07	0.08	0.05
EIMKB008	43	M	0.23	0.20	0.05	0.09	0.00	0.10	0.15	0.15	0.03
EIMKB009	42	M	0.20	0.16	0.21	0.03	0.00	0.07	0.15	0.31	0.05
EIMKB010	48	M	0.27	0.14	0.00	0.04	0.00	0.03	0.06	0.02	0.01
EIMKB011	38	M	0.47	0.43	0.15	0.06	0.43	0.13	0.76	0.23	0.05
EIMKB012	25	M	0.32	0.14	0.00	0.16	0.00	0.01	0.10	0.14	0.04
EIMKB013	26	M	0.44	0.28	0.08	0.05	0.00	0.94	0.62	0.27	0.05
EIMKB014	26	M	0.80	0.67	0.40	1.24	0.11	1.48	2.90	1.82	0.09
EIMKB015	33	M	0.24	0.33	0.00	0.09	0.00	0.10	0.12	0.03	0.03
EIMKB016	26	M	0.40	0.20	0.00	0.00	0.00	0.03	0.05	0.06	0.12
EIMKB017	36	M	0.35	0.20	0.01	0.00	0.00	0.05	0.15	0.04	0.01
EIMKB018	20	M	0.66	0.73	0.88	0.65	0.19	0.85	1.01	0.74	0.12
EIMKB019	23	M	0.21	0.16	0.00	0.00	0.00	0.02	0.05	0.04	0.01
EIMKB020	31	M	0.90	1.58	1.21	0.25	0.34	0.24	2.22	0.90	0.09
EIMKB021	32	M	0.24	0.21	0.14	0.26	0.00	0.05	0.12	0.20	0.05
EIMKB022	28	M	0.26	0.36	0.02	0.00	0.00	0.08	0.18	0.04	0.04
EIMKB023		M	0.36	0.37	0.02	0.04	0.01	0.31	0.16	0.25	0.04

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EIMKB024	29	M	0.73	0.47	0.20	0.11	0.13	0.17	0.17	0.09	0.06
EIMKB025	31	M	0.29	0.41	0.04	0.05	0.23	0.11	0.34	0.11	0.09
EIMKB026	27	M	0.83	1.29	0.52	0.52	0.25	0.38	1.59	0.88	0.47
EIMKB027	48	M	0.87	0.98	0.83	0.35	0.30	0.14	1.55	0.32	0.18
EIMKB028	36	M	1.53	0.82	0.19	0.23	0.08	0.63	0.88	0.85	0.92
EIMKB029	31	M	0.24	0.17	0.31	0.14	0.00	1.33	0.58	0.58	0.03
EIMKB030	38	M	0.91	0.51	0.15	0.16	0.00	0.97	0.87	0.79	0.02
EIMKB031	35	M	0.19	0.17	0.00	0.00	0.00	0.07	0.89	0.05	0.02
EIMKB032	24	M	0.20	0.15	0.19	0.33	0.00	0.84	0.14	1.28	0.03
EIMKB033	24	M	1.04	1.67	1.12	1.38	0.98	1.20	2.57	1.34	0.13
EIMKB034	18	M	0.21	0.10	0.00	0.00	0.00	0.00	0.05	0.02	0.02
EIMKB035	28	M	0.44	0.18	0.14	0.05	0.00	0.23	0.10	0.17	0.06
EIMKB036	32	M	0.13	0.07	0.02	0.08	0.00	0.13	0.27	0.24	0.12
EIMKB037	49	M	0.37	0.41	0.43	0.04	0.32	0.26	0.34	0.78	0.06
EIMKB038	41	M	0.15	0.44	0.00	0.00	0.00	0.10	0.20	0.04	0.02
EIMKB039	31	M	0.25	0.41	0.02	0.00	0.00	0.09	0.22	0.07	0.02
EIMKB040	34	M	0.33	0.32	0.04	0.00	0.08	0.07	0.15	0.08	0.06
EIMKB041	31	M	0.48	0.44	0.06	0.29	0.55	0.29	0.76	0.13	0.06
EIMKB042	40	M	0.25	0.60	0.05	0.02	0.00	0.08	0.61	0.11	0.27
EIMKB043	27	M	0.30	0.00	0.14	0.26	0.00	0.45	0.20	0.23	0.04
EIMKB044	33	M	0.21	0.64	0.03	0.06	0.00	0.15	0.45	0.20	0.07
EIMKB045	36	M	0.27	0.15	0.00	0.00	0.00	0.12	0.08	0.04	0.03
EIMKB046	19	M	0.58	0.31	0.00	0.01	0.00	0.14	0.24	0.02	0.02
EIMKB047	26	M	0.33	0.28	0.02	0.17	0.00	0.14	0.28	0.06	0.05
EIMKB048	29	M	0.23	0.12	0.29	0.10	0.00	1.47	0.64	0.75	0.03
EIMKB049	30	M	1.16	0.32	0.30	0.50	0.00	0.47	0.74	0.27	0.08
EIMKB050	21	M	0.29	0.06	0.00	0.00	0.00	0.04	0.80	0.56	0.03

Table A 3. Growth inhibitory activity of purified IgG from semi-immune adults against seven parasite lines

	Average invasion efficiency (%)						
	3D7	W2mef	K1	GB4	EIMK244	EIMK239	EIMK084
Non-Immune European	102.46	95.83	94.37	135.90	95.45	98.51	97.37
KB01	81.15	54.17	59.15	53.85	68.18	46.27	73.68
KB02	89.75	58.33	81.69	123.08	86.36	86.57	84.21
KB04	88.52	54.17	90.14	115.38	95.45	95.52	89.47
KB05	88.52	58.33	80.28	97.44	95.45	83.58	81.58
KB06	91.39	64.58	76.06	107.69	90.91	97.01	97.37
KB08	81.97	43.75	77.46	66.67	72.73	77.61	94.74
KB09	92.62	58.33	105.63	102.56	86.36	95.52	94.74
KB10	92.21	54.17	105.63	115.38	140.91	116.42	102.63
KB12	93.85	52.08	81.69	110.26	104.55	91.04	102.63
KB13	96.31	70.83	105.63	92.31	81.82	88.06	102.63
KB14	85.66	50.00	66.20	100.00	77.27	32.84	68.42
KB15	84.43	58.33	94.37	128.21	100.00	67.16	76.32
KB16	100.82	60.42	92.96	120.51	100.00	94.03	94.74

KB18	83.61	50.00	80.28	100.00	86.36	56.72	81.58
KB19	97.13	62.50	118.31	117.95	100.00	85.07	92.11
KB20	64.34	50.00	78.87	79.49	90.91	53.73	78.95
KB21	101.23	58.33	81.69	133.33	100.00	82.09	113.16
KB23	95.49	60.42	100.00	128.21	104.55	98.51	121.05
KB24	82.38	56.25	84.51	115.38	81.82	79.10	97.37
KB27	86.48	60.42	90.14	110.26	100.00	19.40	92.11
KB31	92.21	58.33	84.51	120.51	100.00	97.01	81.58
KB33	61.07	41.67	47.89	97.44	72.73	37.31	86.84
KB36	105.33	58.33	107.04	120.51	95.45	86.57	92.11
KB37	97.54	70.83	102.82	112.82	100.00	104.48	86.84
KB39	93.85	52.08	111.27	130.77	86.36	85.07	92.11
KB40	75.82	47.92	83.10	94.87	86.36	67.16	78.95
KB41	84.02	47.92	100.00	112.82	86.36	97.01	81.58
KB43	100.00	56.25	119.72	130.77	95.45	88.06	107.89
KB44	84.43	54.17	111.27	112.82	100.00	94.03	78.95
KB45	85.66	50.00	85.92	115.38	90.91	83.58	100.00
KB46	98.36	62.50	102.82	141.03	109.09	114.93	100.00

KB48	90.98	62.50	100.00	120.51	77.27	89.55	89.47
KB49	73.36	50.00	80.28	79.49	77.27	68.66	73.68
KB50	100.00	66.67	77.46	123.08	81.82	100.00	89.47
KB54	101.23	58.33	101.41	123.08	81.82	92.54	97.37
KB55	100.00	79.17	100.00	123.08	100.00	100.00	86.84
UT	100.00	100.00	100.00	100.00	100.00	100.00	100.00