Maternally transferred antibody levels and IgG3 hinge region length polymorphisms in the risk of clinical malaria in infants in a birth cohort at Kintampo, Ghana.

By

Opoku-Mensah Jones

(ID NO.:10362797)

THIS THESIS IS SUBMITTED TO THE UNIVERSITY OF GHANA, LEGON IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE AWARD OF A MASTER OF PHILOSOPHY IN IMMUNOLOGY.

DEPARTMENT OF PATHOLOGY, UNIVERSITY OF GHANA MEDICAL SCHOOL, COLLEGE OF HEALTH SCIENCES

UNIVERITY OF GHANA, LEGON

JULY, 2014.

To everything there is a season, and a time to every purpose under the heaven: A time to be born, and a time to die; a time to plant, and a time to pluck up that which is planted; A time to kill, and a time to heal; a time to break down, and a time to build up; A time to weep, and a time to laugh; a time to mourn, and a time to dance; A time to cast away stones, and a time to gather stones together; a time to embrace, and a time to refrain from embracing; A time to get, and a time to lose; a time to keep, and a time to cast away; A time to rend, and a time to sew; a time to keep silence, and a time to speak; A time to love, and a time to hate; a time of war, and a time of peace. What profit hath he that worketh in that wherein he laboureth? I have seen the travail, which God hath given to the sons of men to be exercised in it. He hath made every thing beautiful in his time: also he hath set the world in their heart, so that no man can find out the work that God maketh from the beginning to the end. Ecclesiastes 3:1-11 KJV.

DEDICATION

I dedicate this research work to the almighty God Elohim for His grace that was sufficient for me throughout my academic life. I also dedicate this thesis to a woman I call Mummy with all my heart, HerGrace Lady Bishop Apostle Dr Lorraine Rejoyce Laryea my shepherd and also to my Beloved wife Regina Opoku-Mensah. Also to my supervisors for mentoring me in this research work; Prof Daniel Dodoo and Dr Kwaku Poku Asante. This mentoring does not end here but as long as we live in the field of research.

DECLARATION

I hereby declare that, with the exception of quoted articles and references, this project work was duly carried out by me and the results obtained herein are true reflection of the work done under supervision. To the best of my knowledge, this work has neither in part nor in whole been submitted elsewhere for the award of any degree.

……

(Jones Opoku-Mensah)

STUDENT

DATE ……

SUPERVISORS

…… ……

(Prof. Daniel Dodoo) (Dr Kwaku Poku Asante)

DATE …… DATE……

ACKNOWLEDGEMENT

My profound gratitude goes to my supervisors, Prof Daniel Dodoo and Dr Kwaku Poku Asante for their diverse contributions in diverse ways to spearhead this research and for mentoring me in my academic life. I am very grateful to Bright Adu, Akua Agyeman, Nicholas Amoako and Samuel Assafuah for their enormous contribution in pulling out the protocols and optimisation of molecular biology protocols to be used in this research work. To Dr Linda Amoah and Dr Asamoah Kusi and Dr Michael Ofori all at the Department of Immunology, Noguchi Memorial Institute for Medical Research (NMIMR) for their free consultations. I acknowledge Michael Theissen for assisting me with some reagents for my work. My appreciation also goes to Kakra Dickson, Eric Kyei Baffour and Joann Mbroh all at the Immunology Department, NMIMR for their immense contribution in the running of ELISAs. To all my lecturers at School of Medical Sciences- Korle Bu and Noguchi Memorial Institute for Medical Research, especially Prof Andrew Anthony Adjei for training me, giving me the opportunity to lecture and many more a mentor can ever give his apprentice Prof you gave all.

My sincerest thanks goes to my Mummy Rev Dr Lorraine for Her time and love for me and my wife for her support, care and encouragement.

As in publications, the last author is a very important position, therefore above all the appreciations and acknowledgement, let HIM alone, the Creator and the Giver of life, HE who chooses to have mercy on those HE chooses to have mercy take Glory, even Jehovah. YOU who knit me together in the womb and formed my bones, eyes, nerves, brains, muscle, lungs and heart from a single sperm and ovum take YOUR Glory for this work.

ABBREVIATIONS

Ab	Antibody
ADCI	Antibody Dependent Cellular Inhibition
AIA	Afro-Immuno Assay
AMA1	Apical Membrane Antigen
ANOVA	Analysis Of Variance
A-T	Adenine – Thiamine
ACTs	Artemisinin-based combination therapies
Bp	Base Pairs
BEC	brain endothelial cells
BSA	Bovine Serum Albumin
BCR	B cell receptor
CSA	Chondroitin Sulphate A
CD	Cluster of Differentiation
CDC	Centre for Disease Control
CM	Cerebral malaria
CSP	Circumsporozoite protein
DCs	Dendritic Cells
DNA	Deoxyribonucleic Acid
dNTPs	Deoxyribonucleotide Triphosphates
EBA175	Erythrocyte binding antigen
ELISA	Enzyme Linked Immunosorbent Assay

Fab	Fragment, Antigen Binding
Fc	Fragment Crystallizable
FcγR	Fc Gamma Receptor
GHS	Ghana Health Service
GLURP	Glutamate-rich protein
GIA	Growth Inhibition Assay
G6PD	Glucose 6 Phosphate Dehydrogenase
HLA	Human Leucocyte Antigen
HRPO	horseradish peroxidase
Ig	Immunoglobulin
IS	Immune system
ICAM	Intercellular Adhesive Molecule
iRBCs	Infected Red Blood Cells
ITAM	Immunoreceptor Tyrosine Activation Motif
ITIM	Immunoreceptor Tyrosine Inhibitory Motif
IFN-γ	Interferon Gamma
IL-12	Interleukin 12
iNOS	inducible Nitric Oxide Synthase
IEC	Independent or Institutional Ethics Committee
IPT	Intermittent Presumptive Therapy
IRB	Institutional Review Board
ITNs	Insecticides Treated Nets
Kb	Kilobases

KDSS	Kintampo Demographic Surveillance System.
KHRC	Kintampo Health Research Centre
LSA1	Liver-stage Antigen 1
MHC	Major Histocompatibility Complex
MF	Macrophages
MBL	Mannose-Binding Lectin
mIg	Membrane immunoglobulin
MSP	Merozoite Surface Protein
MOH	Ministry of Health
Min	Minutes
Ml	Millilitres
NK	Natural Killer
NO	nitric oxide
NIH	National Institutes of Health
NMCP	National Malaria Control Programme
NMIMR	Noguchi Memorial Institute for Medical Research
OD	Optical density
*Pf*EMP1	*Plasmodium falciparum* Erythrocyte membrane protein 1
PBS	Phosphate Buffer Saline
PBMCs	Peripheral Blood Mononuclear Cells
PCR	Polymerase Chain Reaction
PI	Principal Investigator
QMSC	Qiagen Mini Spin Column

RBC	Red Blood Cells
rpm	Revolutions per minute
Sec	Seconds
SERA	Serine repeat antigen
sIg	Surface immunoglobulin
SOP	Standard Operating Procedure
SSP2	Sporozoite Surface Protein 2
TLR	Toll-Like Receptor
TMB	3, 3', 5, 5'-Tetramethylbenzidine
TNF-α,	Tumour necrosis Factor -alpha.
UM	Uncomplicated Malaria
Ul	Microliters
VCAM1	Vascular Cell-Adhesion Molecule 1
WBCs	White Blood Cells
WHO	World Health Organization

TABLE OF CONTENT

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

Introduction: *Plasmodium falciparum* malaria remains a global public health threat especially for children under five years. Fetuses receive maternal immunoglobulins in utero by passive transfer and this is believed to protect infants at least for the first six months after delivery. IgG3 among the IgG subclasses is known to be more protective because of the long hinge region making the molecule flexible and easier to link antigens and Fc receptors for antigen elimination. However, there are hinge region polymorphisms among the IgG3 molecules that may have an impact on their protective potential.

Aim: This study investigated the relationship between maternally transferred total IgG (IgG) levels and their subclasses against GLURP R0 and MSP1$_{-19}$. Also to investigate the role of infants' IgG3 hinge region length polymorphisms in the risk of clinical malaria in a birth cohort at Kintampo, Ghana.

Methodology: Serum and blood blots samples with the clinical data of participants were taken from a previous birth cohort study conducted in Kintampo. Serum samples were taken from cord blood at birth (month 0), month 3 and month 6 from 202 infants for immunoglobulins level measurement against GLURP R0 and MSP1-$_{19}$ using indirect ELISA. IgG with its subclasses were measured at month 0 and IgG3 levels measured at months 3 and 6. One hundred and forty blood blots were selected to determine infants' IgG3 hinge region length polymorphisms using polymerase chain reaction (PCR).

Results: Among 202 infants, 112 (55.45%) were not protected from clinical malaria (presence of parasites and fever), 68 (33.66%) had asymptomatic parasitaemia (protected) and 22 (10.89%) had no parasites and no fever (indeterminate group). There were

significant differences in anti-GLURP R0 and anti-MSP1-19 total IgG levels at birth ($p < 0.05$) between protected and non-protected infants but not so for the subclasses. There was a sharp decrease in IgG3 levels against both antigens from month 0 to month 3.

Among the 138 infants whose IgG3 hinge region length polymorphisms (IgG3HRLPs) were genotyped, 93.33% had clinical malaria in first year of life. Four IgG3HRLP genotypes were found. The homozygote medium (MM) polymorphism had the highest frequency of 53.33%, followed by the homozygote long (LL) polymorphism with a frequency of 42.22%. The homozygote short (SS) and heterozygote long-medium (LM) polymorphisms were very few among these infants.

Conclusion: Maternally transferred anti-GLURP R0 and anti-MSP1-19 IgG levels at birth were associated with protection against clinical malaria in infants but the subclasses were not. Infants' IgG3HRLPs was not associated with protection from clinical malaria after one year.

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background

Malaria is a preventable and treatable mosquito-borne infectious disease caused by an eukaryotic protist of the genus *Plasmodium*. The species that cause human malaria includes *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium ovale* and *Plasmodium malariae* (Sutherland *et al.*, 2010). According to the World Health Organization's World Malaria Report the estimated number of malaria cases worldwide rose from 233 million in 2000 to 244 million cases in 2005 but gradually declining (WHO, 2010).

Control measures against malaria transmissions include prompt diagnosis and treatment, vector control, and focused research (WHO, 2010). The fear of increasing antimalarial drug resistance, with the decreasing efficacy of vector control interventions in some parts of the world makes the development of effective malaria vaccines an immediate priority (Ballou, 2007, WHO, 2011a). Resistance to the artemisinin drug was confirmed on the Cambodia-Thailand border in 2009 (Aregawi *et al.*, 2009) and is also being suspected in some parts of Myanmar and Vietnam (WHO, 2011b). Vaccines are very important in public health interventions and the development of an effective malaria vaccine would complement efforts and ensure malaria control. An effective malaria vaccine may protect against infection, mild or severe disease or reduce transmission in endemic regions as has been observed with other diseases such as small pox (Centre for Disease Control, 1999). Natural antibodies against blood-stage parasitaemia/antigens have been protective for

people living in endemic areas hence vaccines against blood-stage antigens have been suggested (Stanisic *et al.*, 2009, Fowkes *et al.*, 2010). Malaria vaccine development are ongoing in many parts of the world (Hviid, 2007, Kanoi and Egwang, 2007) which would require in-depth understanding of the immune responses to the malaria parasites.

The immune system is a diffuse, complex network of interacting organs, tissues, certain cells and cell product that protect the body from pathogens and foreign substances, destroys infected and malignant cells, and removes cellular debris. Individuals with deficiencies in the immune system generally succumb to these infectious diseases and malignancies (Zabriskie, 2009). The immune system is divided into two different types of responses, basically the innate and adaptive responses which work in tandem. The innate immunity responses to a pathogen are general protective mechanisms which does not depend on prior encounter with pathogens, has no memory and is quicker in pathogen destruction (Grady, 1988). Cells of the innate system such as phagocytes (macrophages, neutrophils, and dendritic cells), mast cells, eosinophils, basophils, and natural killer cells can directly kill malaria parasites (Zabriskie, 2009). The adaptive responses involves lymphocytes, their associated cytokines and antibodies. T lymphocytes secrete cytokines (interferons and tumor necrosis factors) that inhibits parasites growth and destruction.

B lymphocytes or cells upon encounter with parasites or their antigens are stimulated to proliferate, differentiate into memory cells and plasma cells which secrete antibodies. Five antibody classes (isotypes) are found in the serum: IgG, IgM, IgA, IgE and IgD (Harlow and Lane, 1988). High IgG levels have been associated with protection from malaria (Nebie *et al.*, 2008, Dodoo *et al.*, 2011) in people living in malaria endemic regions. Fetuses receive maternal immunoglobulins in utero by passive transfer across the placenta

(Simister *et al*., 1996) and this is believed to protect infants at least for the first few months after delivery (Akanmori *et al*., 1995). Antibodies, particularly IgG are known to neutralize parasites or lead to parasite killing by macrophages. IgG has four subclasses, IgG1, IgG2, IgG3 and IgG4. IgG3 among these subclasses is known to be more protective because of the long hinge region making the molecule flexible and easier to link antigens and Fc receptors for antigen/parasite elimination (Redpath *et al*., 1998). Protection has been associated with high levels of IgG3 against blood-stage parasite antigens such as glutamine rich protein R0 (GLURP R0) and merozoites surface protein 1_{-19} ($MSP1_{-19}$) (Holder, 1996). These antibodies block merozoites, inhibits erythrocyte invasion, rosette formation and cytoadherence of parasitized erythrocytes to vascular endothelium, leading to a reduced risk of cerebral malaria (Riley *et al.*, 2001). However, there are hinge region polymorphisms which may have influence on the protective role IgG3.

1.2 Problem Statement

P. falciparum resistance to artemisinin is becoming a big threat to humanity since the confirmation of resistance on the Cambodia-Thailand borders (Aregawi *et al.*, 2009). The absence of effective vaccine (Ballou, 2007) and the fear of increasing drug resistance (WHO, 2011b) is raising panic in malaria endemic regions. There is therefore an urgent need for an effective malaria vaccine which require the understanding of the immune responses against the parasites and parasite antigens. There is the need to expand the knowledge on the host immunogenetics which may aid in understanding the various interactions of polymorphic genes with the parasites.

1.3 Justification

This study will add to knowledge which of the maternally transferred IgG subclasses confers more protection against clinical malaria in infants. Most importantly, understanding the immunology of malaria would bring forth better ways to develop possible vaccine candidates. Since antibody levels and hosts' genetics have impact on malaria outcomes, this study will show which IgG3 polymorphism is/are more potent in enhancing protection from clinical malaria in the early years of life where susceptibility is high. Also much work has been done on the parasite and parasite-genetics but not much work has been done in the area of hosts' immunogenetics in this study area where vaccine trials take place. Findings from this study may be useful in future clinical trials. The results may help decide if monoclonal antibody therapy could be an option for passive treatment against clinical malaria in the absence of effective vaccines and the presence of increasing drug resistance.

1.4 Hypothesis

The risk of clinical malaria in infants is influenced by levels of maternally transferred malaria specific antibodies and hinge region polymorphisms in the IgG3 antibody.

1.5 Aim

To determine the role of maternally transferred malaria specific antibodies and the role of infants' IgG3 hinge region polymorphisms in the risk of clinical malaria.

1.6 Objectives

1. To determine the levels of maternally transferred IgG and IgG subclasses (IgG1, IgG2, IgG3 and IgG4) against GLURP R0 and MSP1-19 in cord blood (month 0) by indirect ELISA.
2. To determine the levels of IgG3 to GLURP R0 and MSP1-19 in infants at month 3 and month 6 by indirect ELISA
3. To determine the hinge region length polymorphisms of IgG3 produced in infants at or beyond 12 months

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Malaria

Malaria is an infectious disease caused by a eukaryotic protist of the genus *Plasmodium*. Four species of Plasmodium have been known to cause human malaria, namely *Plasmodium falciparum Plasmodium vivax*, *Plasmodium ovale* and *Plasmodium malariae* (Sutherland *et al.*, 2010, Steketee and Campbell, 2010, Snow *et al.*, 2005). Two simian parasites, *Plasmodium knowlesi* (Cox-Singh *et al*., 2008) and *Plasmodium Cynomolgi* (Ta *et al*., 2014), have also recently been shown to cause human malaria. Of all parasites that currently infect humans, *P. falciparum* infection is the most severe and can result in disease complications. The parasite is transmitted mainly by the bite of infected female anopheles mosquito. The disease results from the multiplication of *Plasmodium falciparum* parasites within red blood cells (Chotivanich *et al*., 2000), causing symptoms that typically includes headache, chills, increased sweating, back pain, myalgia, diarrhea, nausea, vomiting (Eliades *et al.*, 2005). Sometime the disease progresses to coma or death in severe cases (Taylor *et al*., 2004). Children who do not die may suffer brain damage or experience cognitive and learning deficits (Murphy and Breman, 2001). However, it is commonly curable with prompt and effective treatment (Rai and Abraham, 2012).

The disease is widespread in tropical and subtropical regions, including Sub-Saharan Africa, Asia, and Latin America (Martens *et al.*, 1999). In 2010, it was estimated that 3.3 billion people were risk of malaria, however about 216 million malaria cases were reported,

81% of these cases were in Africa resulting in 655 000 deaths. Approximately 86% of these deaths were children under 5 years of age, with 91% of the deaths occurring in African (WHO, 2011a).

Pregnant women and children under 5 years are most susceptible to malaria. This is because during Pregnancy there is a transient depression of cell-mediated immunity of women that allows fetal allograft retention (Meeusen *et al*., 2001, Fievet *et al*., 1995) whiles children under 5 years may have less exposures to build immunity. Pregnancy induced immunosuppression can persist until six months after delivery (Diagne *et al*., 2000). Placental malaria is characterized by the sequestration of *P. falciparum*-infected erythrocytes in placental intervillous spaces (Fievet *et al*., 2002, Maubert *et al*., 2000). Placental malaria is often associated with fetal abortion, stillbirth, and low birth weight of the offspring (McGregor *et al*., 1983).

Malaria infections are routinely confirmed by examination of blood film under light microscope. Each species has distinctive physical characteristics that are apparent under a microscope. Thick blood smears are generally superior for the detection of parasites and thin smears for species identification (Mishra *et al*., 2007). Immunochromatographic dipsticks based on antigen detection are also available for rapid diagnosis (Ochola *et al*., 2006, Jelinek *et al*., 1999). PCR is also available but quite expensive compared to the conventional methods and not often used unless for research purposes (Snounou and Singh, 2002, Mishra *et al*., 2007). Placental malarias are diagnosed by histopathology using placental biopsies.

In the past years, the increase of malaria interventions programmes have resulted in considerable reductions in morbidity and mortality in some parts of Africa (Sutherland *et al.*, 2010, O'Meara *et al.*, 2010). Malaria transmission interventions have included the use of mosquito nets, insect repellents and insecticide sprays. Artemisinin-based combination therapies (ACTs) are now recommended as first line treatments for uncomplicated malaria caused by *P. falciparum* (Davis *et al.*, 2005, Breman *et al.*, 2007). Despite these interventions, there seems to be signs of failure due to drug resistance. Resistance to ACT was confirmed on the Cambodia-Thailand border in 2009 (Aregawi *et al.*, 2009) and resistance are also being suspected in parts of Myanmar and Viet Nam (WHO, 2011b). The fear of increasing drug resistance, with the decreasing efficacy of vector control interventions in some parts of the world (Ballou, 2007, WHO, 2011a) calls additional interventions tools (drugs and vaccines).

2.2 Malaria Problem in Ghana

Malaria is the number one cause of morbidity, accounting for approximately 38% of all outpatient illnesses, 36% of all admissions, and 33% of all deaths in children less than five years. Between 3.1 and 3.5 million cases of clinical malaria are reported in public health facilities each year, of which 900,000 cases are in children under five years (Afudego, 2012). The transmission of malaria in the forest-savanna region of central Ghana is high and perennial. In an entomological study conducted at Kintampo, the main vectors for malaria transmission were *Anopheles funestus* and *Anopheles gambiae*, with an entomological inoculation rate of 269 infectious bites per person per year (Owusu-Agyei *et al.*, 2009).

Severe forms of malaria in Ghana are cerebral malaria and severe malaria anaemia (Mockenhaupt *et al.*, 2004). Cerebral malaria predominantly can result into coma, convulsions, loss of stimulus and hyperthermia and is often fatal (Belnoue *et al*., 2002) whiles severe malaria anaemia results in extremely low haemoglobin levels due to haemolysis caused by the parasites invasion (Abdalla *et al*., 1980). Cerebral malaria which is a neurological manifestation due to the sequestration of *P. falciparum* infected RBCs in the cerebral microvasculature is the severest complication of *P. falciparum* infection (Belnoue *et al*., 2002). It is a major cause of death in children from 2 to 4 years of age (Adu, 2010). Cerebral malaria is known to be in part an immune-mediated disease in which immunological priming occurs during first infection, eventually leading to immunopathology on re-infection (Artavanis-Tsakonas *et al*., 2003).

The disease has a crippling effect on economic growth and perpetuates the cycle of poverty. A study conducted in Northern Ghana revealed that the cost of malaria cure was about 34% of the income of poor households (Akazili *et al.*, 2008).

2.3 *Plasmodium falciparum* biology

The *Plasmodium* species has a complex life cycle. Infection begins with a bite from an infected female *Anopheles* mosquito releasing sporozoites into the host during feeding on humans (Bray and Garnham, 1982). The released sporozoites invade hepatocytes and establish the liver-stage of the infection (Figure 2.1). In the liver stage sporozoites differentiate and undergo asexual multiplication resulting in the release of thousands of merozoites which burst out of liver cells (Meis *et al*., 1983). One infected hepatocyte often

yields between 4000 - 40,000 merozoites (Witney *et al.*, 2002). The blood-stage or erythrocytic stage results when there is invasion of erythrocytes by the merozoites released from the liver (Lambros and Vanderberg, 1979) (Figure 2.1). The duration of the erythrocytic stage of the parasites' life cycle is dependent on the parasite species (Bray and Garnham, 1982)

The trophozoites develop further and reproduce by invading more red blood cells. Destruction of red cells by the erythrocytic stage infection by merozoites result in anaemia, since the bone marrow cannot compensate for the rate at which the red cells are damaged. The rupture of red blood cells produces hemozoin which stimulates cytokine release leading to chills and fever (Roberts and Janovy., 2005).

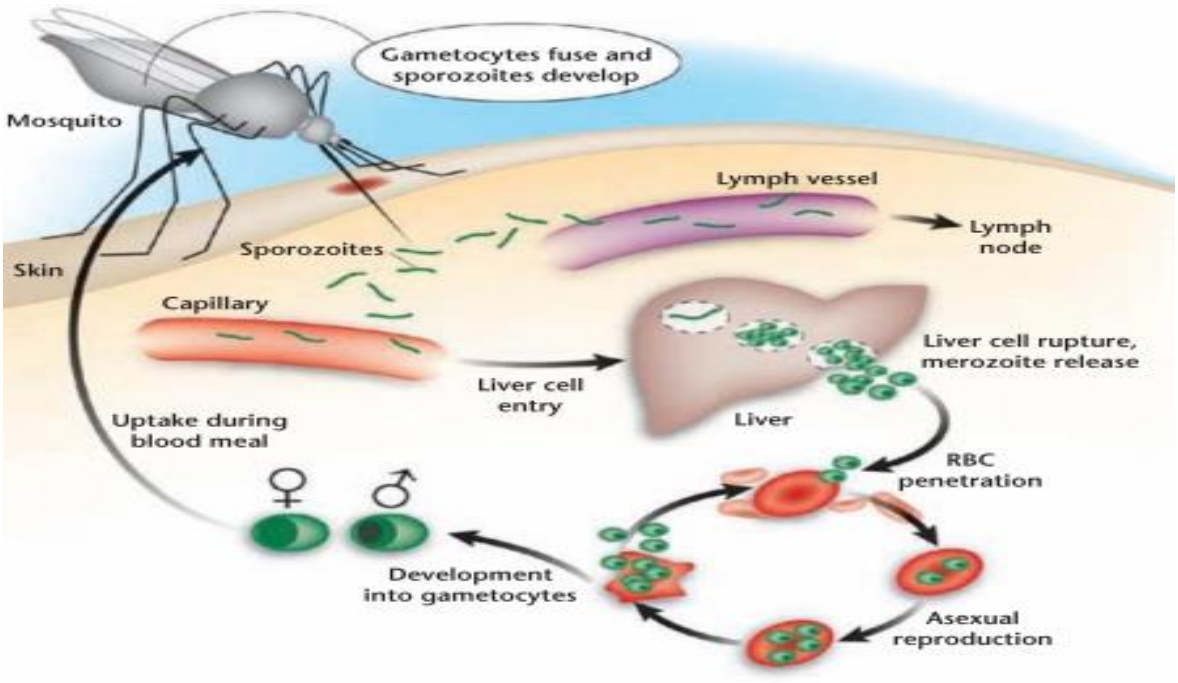


Figure 2.1 life cycle of the malaria parasites (Jones and Good, 2006).

In the erythrocytic stage some merozoites differentiate into sexual forms (male and female gametocytes) (Figure 2.1), which are ingested by the female *Anopheles* mosquito from the human host during a bite (Lambros and Vanderberg, 1979).

In the midgut of the mosquito the male gametocytes undergo cell division to produce several flagellated microgametes which then fertilize the female macrogametes to produce ookinetes. The ookinetes then transverse the mosquito gut wall and encysts on the exterior gut wall as an oocyst. Oocytes then rupture to release hundreds of sporozoites which migrate to the salivary glands of the mosquito. These sporozoites are re-injected into human host to perpetuate the life of the parasite (Bray and Garnham, 1982).

The genome of the *P. falciparum* encodes about 5,300 genes and is composed of 22.8 megabases distributed among 14 chromosomes, excluding introns (Gardner *et al*., 2002). Genes involved in antigenic variation are concentrated in the subtelomeric regions of the chromosomes. *P. falciparum* has a highly variable cluster of genes towards the telomeres which play important role in evasion of the immune system and host–parasite interactions (Gardner *et al*., 2002). The mean length of *P. falciparum* genes is about 2.3 kb which is significantly larger compared to that of other pathogenic organisms which ranges between 1.3 to 1.6 kb (Gardner *et al*., 2002). Epigenetic mechanisms appear to be one of the major mechanisms employed to complete their life cycle and survive in human hosts (Duraisingh *et al*., 2005). These mechanisms, even though not fully explored, have been implicated as one of the key players in antigenic variation which is often employed in immune evasion (Gupta *et al.*, 2013).

2.4.0 The immune system

Living things are under constant attack from disease-causing agents. The immune system plays the role of protecting an organism from infectious agents such as foreign molecules, bacteria, viruses, fungi, etc. out of the body, and to destroy any infectious agents that do invade the body (Abbas *et al*., 1996). To function properly, the immune system must detect a wide variety of agents and distinguish them from the organism's own healthy tissue. Individuals with deficiencies in the immune system generally succumb to infectious diseases and malignancies (Kersey *et al*., 1973). The elimination of malignant cells or tumor antigens, cellular debris and antibodies that attack self are all taken care by the immune system (Zabriskie, 2009).

Organs and tissues involved in the immune system are called the lymphoid organs and include the thymus, bone marrow, lymph nodes, spleen, appendix, tonsils, and Peyer's patches (in the small intestine) (Figure 2.2). The blood vessels and lymphatic vessels are important parts of the lymphoid organs, because they carry lymphocytes to and from different areas in the body. Within lymphoid organs, immune tissues allow for maturation of immune cells, trap pathogens and provide a place for immune cells to interact and mount a specific response (Parham, 2009, Coico and Sunshine, 2009)

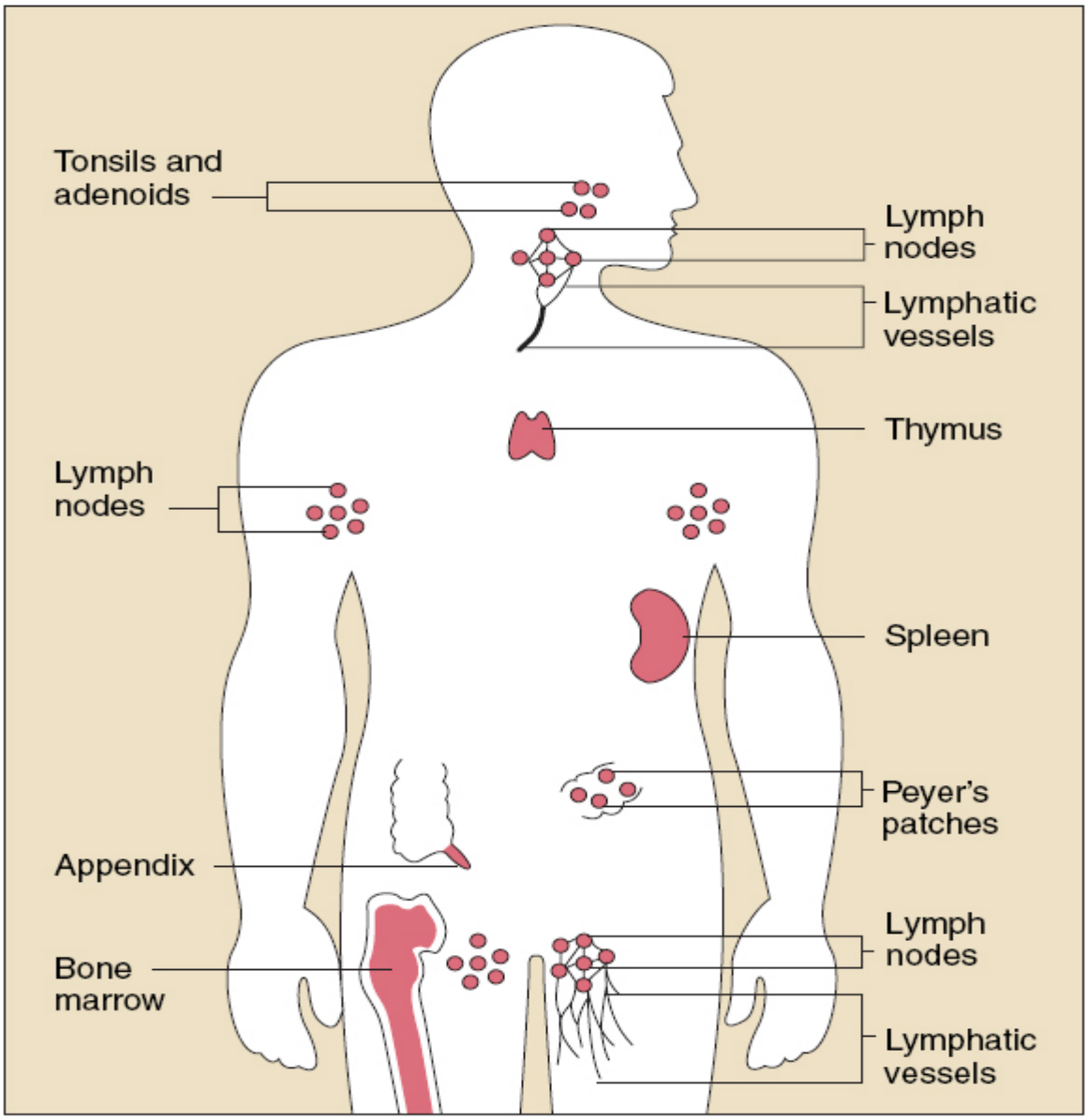


Figure 2.2 The immune system as a diffuse, complex network of interacting tissues *(contains cells and cell product not shown) and organs that protect the body from pathogens and other foreign substances, destroys infected and malignant cells, and removes cellular debris. The system includes the thymus, spleen, lymph nodes and lymph tissue.* Image from http://www.niaid.nih.gov/topics/immuneSystem, 28 February 2013.

The human immune system is classified into two, basically the non-specific (innate) and the specific or acquired (adaptive) immunity (Figure 2.3). Both innate and adaptive systems depend on the ability to distinguish between self (components of an organism's body that can be distinguished from foreign substances) and non-self-molecules, cells or tissues (Grady, 1988).

2.4.1 Non-Specific (Innate) Immunity

Everyone is born with innate (or natural) immunity, a type of general protection which does not depend on prior encounter with pathogens, has no memory and is quicker in pathogen destruction (Grady, 1988). Natural immunity is created by the body's natural barriers, such as the skin and mucous membranes (protective barriers that line the mouth, nose, throat, and gastrointestinal tract the urinary tract, and on the eye surface) (Figure 2.3). These physical barriers are the first line of defense in preventing diseases from entering the body. When there is a breach in the outer barriers, pathogens then gain access to cause harm. Some white blood cells (phagocytes) engulf or inhibit pathogens that gain access due to breach in the outer barriers. Innate immune cells form the second arm of the innate immune system and are important mediators in the activation of the adaptive immune responses (Figure 2.3). These include phagocytes (macrophages, neutrophils, granulocytes, and dendritic cells), mast cells, eosinophils, basophils, and natural killer cells. These cells identify and eliminate pathogens, either by attacking pathogens through contact or phagocytosis (Zabriskie, 2009). Stimulation of the innate immune system is mainly mediated through pattern recognition receptors (PRR), which are conserved molecular structures found in large groups of pathogens (Miller *et al.*, 2014).

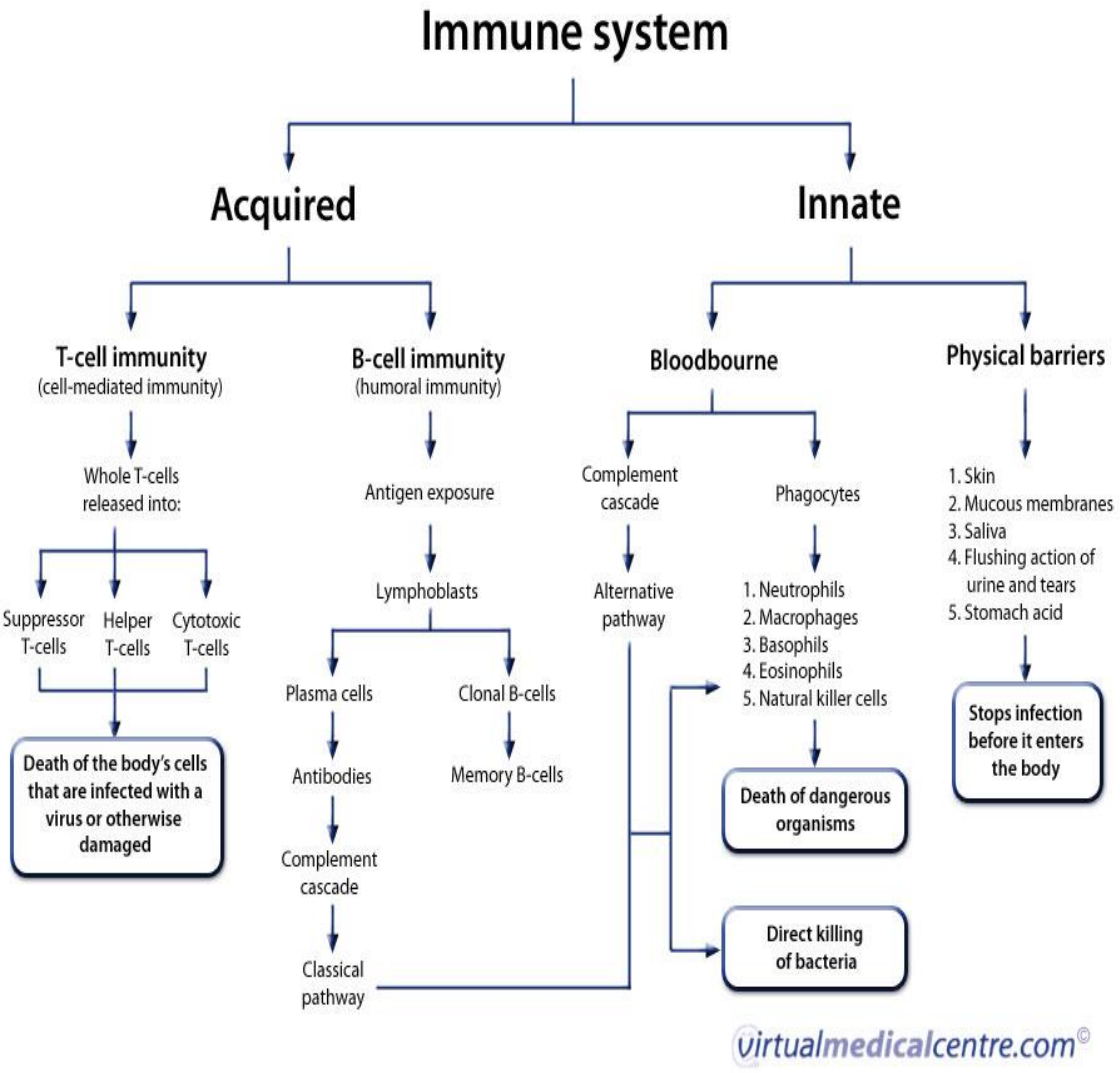


Figure 2.3 A simplified chart on the immune system. Image from http://www.google. com.gh/imgres, 4 January, 2014.

Inflammation is one of the first responses to infection and is characterized by redness, swelling, heat and pain caused by increased blood flow into tissue. The complement system is the major humoral component of the innate response and involves a biochemical cascade of about 20 different proteins that attacks the membranes of foreign cells (Coico and Sunshine, 2009). Antimicrobial substances such as Lysozyme in tears, defensins, spermin,

hydrochloric acid in the stomach also contribute to innate immune function. Dendritic cells as professional antigen presenting cells present processed antigen to activate lymphocytes under the adaptive arm of the immune responses and thus act as a link between the innate and adaptive arms (Grady, 1988).

2.4.2 Specific (Adaptive) Immunity

The adaptive (or acquired) immunity involves lymphocytes and develops as people are exposed to diseases or immunized against diseases through vaccination (Ozer, 2012). Adaptive immunity complements the innate immune system. Specific immunity allows for a targeted response against a specific pathogen. Two types of lymphocytes, T cells and B cells, are vital to the specific immune response (Parham, 2009). Adaptive immunity creates immunological memory after an initial response to a specific pathogen, leading to an enhanced response to subsequent encounters with the same pathogen (Ozer, 2012). Adaptive responses may further be divided into two components, basically, the humoral response and the cellular response to a given antigen (Figure 2.3). Humoral responses produce antibodies in response to a given antigen whiles cellular responses are mediated by the T lymphocytes or their secreted cytokines (Parham, 2009). These immune effectors or components can be transferred from one source to another to induce immunity known as passive immunity which lasts for a short time and has no memory (Zabriskie, 2009).

2.4.3 Activation of T- Cell Responses

Antigen presenting cells (APCs) recognize foreign molecules, engulf them and digest (process) them into smaller molecules without which T cells cannot recognize them. Interaction between APCs and T cells are enhanced by co-stimulators which are molecules on cell surfaces that induce signal transduction upon interaction or bind to each other. Thus CD80 (B7-1) and CD86 (B7-2) on the APC binds to receptors (CD28 and CTLA-4) on the T cell confirms the need for immune response. The absence of these co-stimulators leads to T-cell unresponsiveness and anergy. Processed antigen is presented to the T cells combines with MHC complex present on the surface of APCs. The most efficient APCs are the dendritic cells (Zabriskie, 2009) and have high concentrations of MHC class I and II antigens, co-stimulatory molecules, and adhesion molecules on their surface. The processed antigen closely associated with MHC1 molecules activates T cell responses (Coico and Sunshine, 2009).

T cells act as effector cells and may function as both helpers and suppressors, depending on the stimulus provided by APCs. T cells have receptors known as T-cell receptors (TCR), which provide a means of recognizing self-molecules and effector functions. There are two types of TCRs namely gamma- delta (γδ) TCRs and alpha- beta (αβ) TCRs. These TCRs are associated with the CD3 protein, which transduces the antigen recognition signal into the cell (Zabriskie, 2009). T cells are grouped into T helper cells (CD4+ cells) and T cytotoxic cells (CD8+ cells). T helper cells are sub grouped into T helper 1 (Th1) and T helper 2 (Th2), each having a unique task (Parham, 2009). Th1 cells secrete TNF and IFN-α and mediate cellular immunity. T helper 2 cells when activated release IL4, 5, and 13 to control infections. The presentation of antigens in combination with MHC class I

molecules activates T cytotoxic cells to destroy infected cells (Grady, 1988). Cytotoxic cells also play a role in graft rejection. Regulatory T cells (also called suppressor T cells) controls immune responses (Parham, 2009) by releasing cytokines such as IL-10 and transforming growth factor-β (TGF-β) to negatively regulate both innate and adaptive responses (Abdalla *et al*., 1980).

2.4.4 B cell immunity and antibody production

B cells are primarily responsible for antibody production. These cells express immunoglobulins on their surface as B cell receptor (BCR). In early stages, B cells show intracellular μ-chains and then surface IgM as BCR. Through certain molecular processes, these B cells can later express IgG, IgA, or IgE, a phenomenon known as isotype or class switching (Kracker and Radbruch, 2004). Th2 cells respond to antigen presented by MHC class II molecules as a complex on APC cells. Th2 cells then secrete IL-4, IL-5, IL-10, and IL-13 stimulating B cells proliferation (Zabriskie, 2009) and the formation of terminally differentiated antibody-producing cells known as plasma cells. Plasma cells secrete large quantities of antibodies, which bind to the specific pathogen antigens against which they were produced (Parham, 2009). Some of the proliferated B cells differentiate into memory cells so that on a secondary contact with the same antigen there would be an anamnestic response. Isotype switching is very important in adaptive humoral responses. It is mediated by stimulatory molecules, CD40 on the B cell interacting with CD40L on activated T cells to stimulate B cells to switch from IgM production to other isotypes (Zabriskie, 2009).

2.4.5 Antibody structure

Immunoglobulins, also known as antibodies, are glycoprotein molecules produced by plasma cells (white blood cells). They play critical role in immune response by specifically recognizing and binding to particular antigens, such as pathogens and aiding in their destruction. (Janeway *et al*., 2001). The antibody molecule is made up of four polypeptide chains, comprising of two identical light (L) chains and two identical heavy (H) chains which join together to forms a flexible Y-shaped structure. Both the H and L chains have regions of variable amino acid sequence and a portion with a conserved amino acid sequence known as the constant region (Davies and Metzger, 1983). The L chains are held to the H chains by non-covalent interactions and disulfide linkages (Natvig and Kunkel, 1973). There are two type of the L chain, namely Kappa (k) and lambda (l) L chain. An antibody molecule may have any of the L chain types but not both. The variable (V) region of the light heavy chains pair-up in each arm of the antibody to generate two identical antigen-binding sites (Fab) (Figure 2.4). The presence of two antigen-binding sites allows antibody to cross-link antigens and to bind them much more stably. The "tail or trunk" of the Y shaped antibody molecule called the crystalizable fragment (Fc) is composed of the carboxyl-terminal domains of the heavy chains. Proteases have been used to dissect the structure of antibody molecules to determine the functions of the various parts of the molecule. The hinge region that links the Fc and Fab portions is in reality a flexible tether, allowing independent movement of the two Fab arms (figure 2.4). The Fc regions and hinge regions differ among the different antibody isotypes, and subtypes and thus determine their functional properties (Janeway *et al*., 2001).

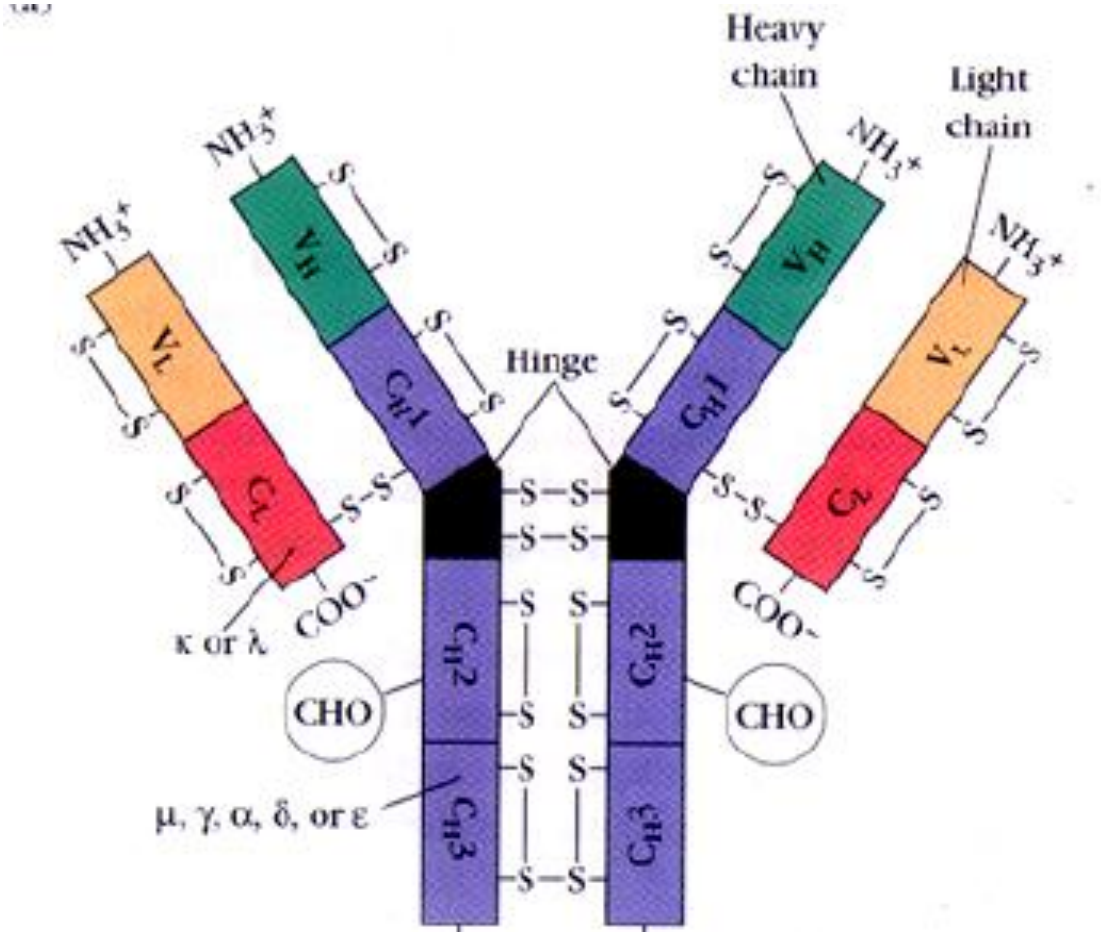


Figure 2.4 General structure of an antibody molecule. Image taken from http://www.google. com.gh/imgres, 4 January, 2014.

2.4.6 Flexibility in Antibody Structure

Flexibility in this context indicates that there is more than one static permissive orientation. That is, the ability to jiggle (rapidly change their position and/or orientation), display a variety of Fab-Fab angles and bending of the Fab-Fc in various angles (Roux *et al.*, 1998). The segmental flexibility of some antibody isotypes play important role in their functionality (Davies and Metzger, 1983). Antigen-induced antibody cross-linking on their

effector cells via cell surface receptors which determines whether an antigen engaged with an antibody will be translated into a signaling event leading to the elimination of the antigen (Leusen and Nimmerjahn, 2013). The immune complexes formed by the various human immunoglobin classes and IgG subclasses visualized with immunoelectron microscopy revealed that differences in hinge flexibilities had a profound effect on the types and sizes of their soluble immune complexes (Tao *et al*., 1991, Janeway *et al*., 2001). The hinge-folding flexibility, which is the variation in the angle between the two Fab arms, is more profound in IgG3 than the other IgG subclasses (Roux *et al*., 1998). There is a correlation between the length of the hinge and Fab-Fc flexibility (Dangl *et al*., 1988, Roux *et al*., 1998).

IgM and IgE have no formal hinge instead they possess an extra C region domain at the hinge site. However, the Fab arms of IgM and IgE are believed to show some limited degree of flexibility at the CH1-CH2 and CH2-CH3 (Figure 2.4) junctions of the immunoglobulin molecule (Beavil *et al.*, 1995).

2.4.7 Antibody classes, properties and functions

There are five immunoglobulin classes (isotypes) found in humans, namely IgG, IgM, IgA, IgE and IgD. The constant domains of the heavy chain define the class and subclass of the antibody (Harlow and Lane, 1988). IgG molecules possess heavy chains known as γ-chains; IgM have μ-chains; IgA have α-chains; IgE have ε-chains; and IgD have δ-chains (Fundenberg *et al*., 1976). The amino acid sequences that confer these functional differences are located within the Fc domain (Solomon and Weiss, 1995). Antibody classes do differ in their valencies, which is the number of arms available to bind antigens. This

arises from the ability of certain immunoglobulins to form multimers through linkage of their Fc domains via a J chain (Fundenberg *et al*., 1976). IgA exits as monomeric and polymeric forms (Davies and Metzger, 1983). It has two main subclasses, IgA1 and IgA2 (Solomon and Weiss, 1995). They form approximately 15% of the total serum Ig. Secretory IgA which are dimers provide the primary defense against local infections because of its abundance in mucosal secretions (e.g., saliva, tears). The principal function of secretory IgA is to prevent passage of foreign substances into the circulatory system (Fundenberg *et al*., 1976).

In human, IgM is the first immunoglobulin class to be synthesized by neonates. Serum IgM exists as a pentamer but is expressed as a monomer on B cell surface (Fundenberg *et al*., 1976) in the Bone marrow. It predominates in primary immune responses and is the most efficient complement fixing Ig (Mold *et al*., 1999). It is approximately 10% of normal human serum Ig content and binding of IgM to a cell/pathogens leads to agglutination. IgM-antigen immune complexes are often destroyed by complement fixation or receptor mediated endocytosis by macrophages (Harlow and Lane, 1988).

IgD is mostly membrane bound and are found on mature naïve B-lymphocytes in lymphoid organs as BCR. IgD concentrations are very low in human serum. It represents about 0.25% of the total serum immunoglobulins (Vladutiu, 2000).

IgE primarily defends against parasitic invasion and is responsible for allergic reactions (Fundenberg *et al*., 1976). IgE concentrations are also very low in the serum, about 0.05% of the total serum immunoglobulins (Winter *et al*., 2000).

IgG is a monomer and is the abundant antibody isotype found in the serum representing approximately 75% of serum immunoglobulins in humans (Harlow and Lane, 1988). Four subclasses of IgG have been identified namely IgG1, IgG2, IgG3 and IgG4 (Schur, 1987). IgG1 and IgG3 fix complement and bind phagocyte Fcγ receptors well whilst IgG2 fixes complement but binds Fcγ receptors poorly. IgG4 does not fix complement effectively. Antibody-mediated immune responses are linked to the cellular effector functions by Fc receptors (FcRs) which constitute a family of glycoprotein complexes consisting of ligand-binding chain and associated signaling chains found on the surface of all immune cell types of the immune system (Ravetch and Bolland, 2001). These FcRs are classified based on the type of antibody isotype they recognize. Those that bind IgG, IgA and IgE are called Fc-gamma receptors (FcγR), Fc-alpha receptors (FcαR) and Fc-epsilon receptors (FcεR) respectively (Raghavan and Bjorkman, 1996).

2.5.0 Immunity to malaria

Natural protective immunity to malaria is in three stages, starting with immunity to life-threatening conditions (severe diseases) and followed by immunity to symptomatic diseases (uncomplicated diseases) and lastly partial immunity to parasite infection (Schofield and Grau, 2005). All these phases of natural immunity to malaria is a complex and slow process (Snow *et al*., 2001). This is because of the diversity of antigens expressed by the parasite antigens and their stimulation of multiple immune responses. Malaria immunity is not fully understood but it is known that both the innate and the adaptive immune mechanisms (cellular and humoral responses) are involved (Riley *et al*., 1994).

2.5.1 Innate immunity to malaria

Immune responses to infectious agents are mainly initiated by the interaction of pathogen-associated molecular patterns (PAMPs) with receptors expressed on host cells. (Abdalla *et al*., 1980). The inoculation of sporozoites, activates γδ-T cells, natural killer (NK) cells and IL-12 production (Wagner *et al*., 2006). Monocytes release TNF-α to inhibit parasite growth and nitric oxide to kill parasites. Monocytes can also phagocytose antibody opsonized parasites (Celada *et al*., 1983).

Liver-stage infection is mainly suppressed by the innate responses involving IFN-driven responses. This involves Natural killer and $CD49b^{+}CD3^{+}$ natural killer T (NKT) and CD1d-restricted NKT cells, which secrete IFNγ are critical in reducing liver-stage burden of a parasite infection. IFN signaling leads to other immune cells recruitment and subsequent parasite elimination (Miller *et al*., 2014). Parasites are identified by immune cells such as dendritic cells, monocyte, NK- and NKT-cell which can kill the parasite directly or by the release of their respective cytokines which may kill or inhibits parasite activities. NK cells are activated upon recognition of *Plasmodium* infected erythrocyte *and* the depletion of NK cells result into increased parasitaemia and increased mortality (Hansen *et al*., 2005).

Blood-stage plasmodium infection generates cascades of innate immune responses that are mediated by interferon γ (IFNγ), TNF α, and interleukin-12 (Riley and Stewart, 2013). These cytokines limits or inhibits parasite growth and replication or even activates other cells of the such as monocytes and macrophages which kill parasites by phagocytosis of opsonized infected erythrocytes (Celada *et al*., 1983).

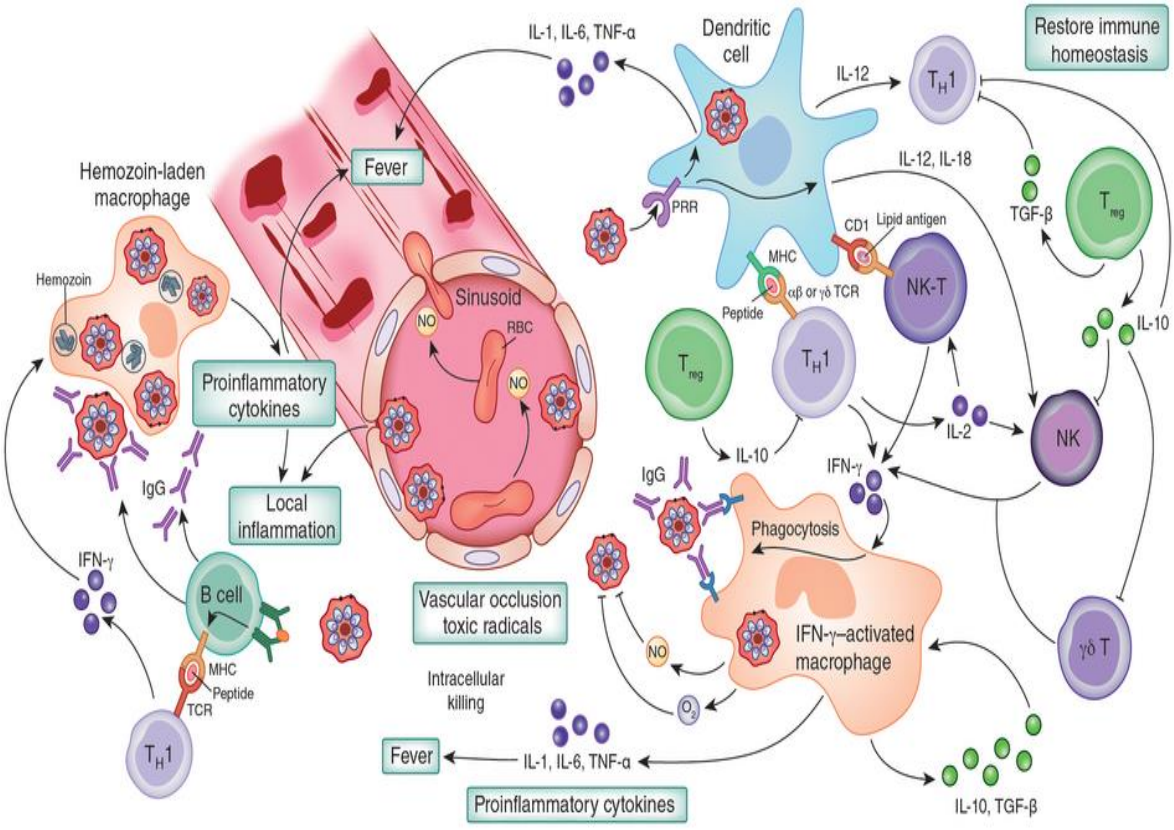


Figure 2.5 A chart showing the innate and adaptive immune responses to malaria
Image from (Riley and Stewart, 2013)

2.5.2 T cell immunity to malaria

Both the CD4+ and CD8+ T cells play important roles in immunity to malaria, but at different stages. During the liver stage CD8+ T cell are most important (Mostov and Deitcher, 1986) with the help of cytokines (IFN-γ, TNF) and other factors, such as nitric oxide (Macpherson and Slack, 2007). The CD4+ T cells are crucial in the immunity against asexual blood stage malaria. Th1 cells are the main cell population responsible for cell-mediated immunity via the activation of macrophages with the release of inflammatory cytokines. They fight intracellular pathogens such as the malaria parasite and controls

primary parasitaemia (Hill *et al*., 1991). Both Th1 and Th2 immune response pathways are crucial for resistance to malaria and the balance between these two cell populations are important in determining the outcome of the infection. T cells bearing γδ TCR plays a major role in immunity to blood stage malaria. Lack of γδ T cells often results in chronic parasitaemia (Seixas and Langhorne 1999) and γδ T cells from the peripheral blood of malaria non-immune individuals can inhibit growth of *P. falciparum* (Troye-Blomberg *et al*., 1999).

Despite all these mechanisms, some organisms such as the malaria parasites have acquired diverse mechanisms for evasion and tricking the immune system that may lead to poor immune responses. These including antigen diversity or polymorphism, clonal antigenic variation and also the ability to modulate the immune response (Hisaeda *et al*., 2005, Millington *et al*., 2006), cause significant immune suppression (Schmid-Hempel, 2009) and total immune suppression (Hisaeda *et al*., 2005). These parasites often live inside hosts ‘cells and are largely hidden from the immune system (Hisaeda *et al*., 2004). Malaria parasites also induces immune suppression, by increased CD4+CD25+ regulatory T cells (Treg) responsiveness (Hisaeda *et al*., 2004).

2.5.3 B cell immunity to malaria

The importance of antibodies in protective immunity against *P. falciparum* infection was demonstrated when passive transfer of IgG from malaria exposed adults had curative effects in children (Cohen *et al*., 1961). These antibodies are mostly involved in antibody

dependent cellular inhibition (ADCI),phagocytosis, lysis of parasite-infected RBCs by monocytes and neutralization of parasite or parasite antigens. Immunoglobulin levels have been shown to generally increase with age and this is associated with a decreased risk of clinical malaria (Dodoo *et al.*, 2011).

Plasmodium parasites secrete soluble antigens, which are important in humoral immune responses (Ramsey *et al*., 2002). Some proteins on the surface of the parasite, such as the blood-stage antigens contribute to the stimulation of the humoral immune response (Tolle *et al*., 1993). Clinical immunity to blood-stage malaria depends on number of exposure to the parasites and age of the host (Dodoo *et al*., 2011) and most importantly on the acquisition of a repertoire of antibodies to different parasite antigens (Dodoo *et al*., 1999). Acquired humoral immunity against *Plasmodium* reduce the density of malaria parasites in the host, and are also able to inactivate gametocytes (Buckling and Read, 2001). Antibodies raised against merozoites surface proteins (MSP), glutamate rich proteins (GLURP) and apical membrane antigen 1 (AMA1) have been found to prevent erythrocyte invasion. These parasite antigens are the targets of invasion-inhibition antibodies and antibody-dependent cellular inhibition antibodies. Soluble antibodies bind cells or antigens bivalently or polyvalently and cross-link to exert their effector function (Ravetch and Clynes, 1998).

2.5.4 Antigen targets of anti-malarial antibodies

Some malaria immunological studies indicated that high titres of antibodies to some antigens are often associated with protection (Nebie *et al.*, 2008, Dodoo *et al.*, 2011).

Antibodies that bind to the surface of the merozoites, and proteins that extend from the apical complex of organelles (involved in erythrocyte recognition and invasion) seem to play important role in immunity to asexual blood stages. These antibodies are thought to neutralize parasites or lead to Fc-dependent parasite killing by macrophages (phagocytosis) (Ravetch and Clynes, 1998). Typical examples of these antigens/proteins are MSP, AMA1 and GLURP. MSP1-$_{19}$ is the C-terminal domain of the MSP which is required for attachment and invasion of erythrocyte by merozoites (Holder, 1996). These antibodies block merozoites, inhibit erythrocyte invasion and cytoadherence of parasitized erythrocytes to vascular endothelium, leading to a reduced risk of cerebral malaria (Riley *et al.*, 2001). Immunizations with GLURP have generated antibodies capable of mediating growth-inhibitory activity against *P. falciparum* in vitro (Theisen *et al.*, 1998, Hermsen *et al*., 2007). Antibodies generated against GLURP and MSP have been reported to have synergistic protective effect in malaria immunity (Soe *et al*., 2004). GLURP R0 and MSP3 in their separate forms as well as in a fused form are readily recognized by the sera of the naturally exposed populations (Theisen *et al*., 2004, Soe *et al*., 2004).

2.6. Cytophilic and non-cytophilic Antibodies

The cytophilic antibodies (IgG1 and IgG3) have been shown to be protective, while non-cytophilic ones (IgG2 and IgG4) have not been associated with protection from clinical malaria (Lusingu *et al*., 2005, Roussilhon *et al*., 2007) . IgG1 and IgG3 are responsible for pathogen clearance via opsonization and activation of NK cells and complement system (Mina-Osorio and Ortega, 2004). Merozoite surface proteins and glutamate-rich protein

have been reported to be the leading targets of cytophilic antibodies effective in antibody-dependent parasite clearance or inhibition (Soe *et al*., 2004, Theisen *et al*., 2004). IgG1 and IgG3 titres formed against these antigens are high in malaria protected people, whereas IgG2 and IgG4 are often high in non-protected individuals (Oeuvray *et al*., 1994, Nebie *et al*., 2008). High titres of cytophilic antibodies against GLURP is associated with reduced risk of febrile malaria whiles high titres of non-cytophilic antibodies against GLURP are not associated with reduction in parasite density (Lusingu *et al*., 2005).

2.7 IgG3 hinge region length polymorphisms in humans

The middle part of the IgG3 heavy chain called the hinge region covalently links the two gamma3 chains to each other (Michaelsen *et al.*, 1977). In some studies, IgG3 has been shown to be superior over IgG1 in parasite clearance, a property which might be linked to the hinge region of IgG3 being longer than that of IgG1 which allows for increased flexibility and ability to link both antigens and FcγRs effector cells (Soe *et al.*, 2004, Nielsen *et al.*, 2007). It is thought that these features of IgG3 may depend on polymorphism within the IgG3 hinge region (Pleass, 2009, Adu, 2010).

The hinge is about four times longer than the hinge regions seen in the other three human IgG subclasses which probably may be due to a quadruplication of a 45-nucleotide DNA segment (Michaelsen *et al.*, 1977, Huck *et al.*, 1989). Besides this structural difference in the hinge region, the IGHG gene is also polymorphic (Huck *et al.*, 1989). The IgG3 hinge is coded for by four exons separated by short introns. The first exon coding for a 17 amino

acid (aa) sequence whiles Exons 2, 3, and 4 are identical, each coding for a 15 amino acid residue similar in sequence (Endo and Arata, 1985). Thus, the gamma3 hinge is made up of 62 amino acid residues and consists of an NH2-terminal 17 amino acid residue segment followed by a 15 amino acid residue segment which is identically and consecutively repeated three times. The NH2-terminal 17-residue segment shows 70% resemblance in structure or sequence with the repetitive 15-residue segment and appears to be the result of a small insertion and several point mutations of the same 45-nucleotide DNA stretch (Michaelsen *et al.*, 1977). The structural IgG3 hinge is composed of a 12-aa upper hinge (UH) stretching from the C-terminal end of CH1 to the first hinge cysteine, a 50-aa middle hinge (MH) stretching from the first to the last cysteine in the hinge, and an 8-aa lower hinge (LH) stretching from the last cysteine (Figure 2.4) (Nezlin, 1990).

A comparative studies between the native and the modified IgG3 hinge mutants shows that mutants that retain their terminal 17-aa segment (17-15-15 and 17-15) were almost as capable as the wild-type IgG3 (17-15-15-15 hinge) in ring dimer formation whiles both 15-15-15 and 15 mutants showed least flexible. There seem to be a direct relationship between the UH length (17 aa portion) and Fab arm flexibility. The 15-15-15 and 15 mutants have a folding which adversely affected Fab-Fab flexibility in these molecules**.** Truncated IgG3 hinges are created by the removal of specific hinge exon(s) and are designated 17-15-15, 17-15, 15-15-15, 15-15 and 15 (Michaelsen *et al.*, 1990).

The hinge of IgG3 has the highest degree of flexibility amongst the IgG subclasses (Burton and Woof, 1992, Dangl *et al*., 1988, Tao *et al.*, 1991) which is thought to have effect on its effectors abilities. IGHG3 polymorphism corresponds to a variable number of exons

coding for the flexible hinge segment of the IgG3 antibody. Thus there exists a 4-exon, 3-exon and 2-exon forms making three forms of phenotypes (Dard *et al.*, 1996). This was not different from the allelic forms found in Ghana by Adu *et al* in a study of a sub-population of Ghanaian children. The most predominant IgG3 hinge region Length polymorphism allele was the long (L). The heterozygous genotype (LM) was the highest frequency and the homozygous medium (MM) being the least present (Adu, 2010).

2.8 Maternally transferred Antibodies

Neonates are relatively protected from clinical malaria and its severe consequences for the first few months of life and this is evident in the higher hospital admissions for malaria in older infants than in infants under 6 months. Maternally derived antibodies are commonly believed to provide protection against many infectious diseases too (Riley *et al.*, 2001). Maternally acquired IgGs have also been discovered to be involved in reducing the severity of *P. falciparum* malaria (Høgh *et al.*, 1995, Mutabingwa *et al.*, 2005). The length of time for this protection is thought to be inversely related to the transmission intensity of malaria parasite but directly related to the starting concentration of antibody at birth (Brabin, 1990, Snow *et al.*, 1998).

The nature of IgG subclasses influence their function (Nimmerjahn and Ravetch, 2008), and it was realized that IgG1 and IgG3 (cytophilic subclasses) form the predominant responses to some antigens; PfRh4 (Reiling *et al.*, 2012), GLURP and MSP (Nebie *et al.*, 2008). Growing evidence suggest that IgG3 is superior over IgG1 in parasite clearance, a property which might be linked to the hinge region of IgG3 being longer than that of IgG1

which allows for increased flexibility and ability to link both antigens and FcγRs effector cells (Soe *et al.*, 2004, Nielsen *et al.*, 2007).

CHAPTER THREE

METHODOLOGY

3.1 Study Design

This cohort study was conducted using clinical data and archived serum samples and blood blots (filter paper blood spot) from the Kintampo Birth Cohort Study (Asante *et al*., 2013). The Kintampo Birth Cohort Study was conducted between August 2008 and September 2010 at the Kintampo Health Research Centre. Pregnant women were identified using the Kintampo Health and Demographic Surveillance System (KHDSS), consented and followed up until delivery. About 3,000 infants were recruited into the Kintampo Birth Cohort Study and followed up for a maximum of 2 years. The study also collected data on other potential confounding variables such as insecticide treated net (ITN) use, and household socioeconomic characteristics.

3.2 Study area

The Kintampo Birth Cohort Study was conducted in the Kintampo North Municipality, the Kintampo South Districts, and the Nkoranza South District. The districts lie within the forest-savanna in the middle belt of Ghana, in the Brong Ahafo region. The mean temperature ranges between 18°C and 38°C and the average rainfall is 1250 mm per annum, occurring mainly between May and October each year. The average parasite prevalence as studied by Owusu-Agyei *et al* (Owusu-Agyei *et al.*, 2009), in all age was 58% and that of children five years and below was 64% in 2004. Children less than 5 years

of age do get as many as seven malaria attacks per child per year. The annual entomological inoculation rate was reported as 269 infective bites per person per year (Owusu-Agyei *et al.*, 2009).

The main birth cohort study recruited approximately 3000 pregnant women. The participants (pregnant women) were followed up on scheduled monthly visits and cord blood samples, placenta tissues and maternal blood samples taken at delivery. The neonates/infants born to these mothers were followed up after birth over a period of two years. Blood samples for malaria slide reading, full blood count, blood blots and plasma samples were obtained from the infants on scheduled visits and any time infants had fever, these samples and others such us blood cultures, urine and stool examinations requested by study clinicians.

3.3 Case definition for clinical malaria

The presence of 5,000 parasites/ul blood in addition to a measured body temperature of ≥37.5°C (fever) (Rogers *et al.*, 2006) was considered as clinical malaria. However, with children ≤ 2 years, a count of 1000 parasites/ul of blood in addition to a body temperature ≥>37.5°C (fever) was considered as clinical malaria since Rogers' values were often used for adult studies. Other potential variables that could affect the risk of clinical malaria such as long-lasting insecticidal nets, season of birth, Socio-economic status and been born to mother with placental malaria were corrected or adjusted for in the statistical analysis. Antibody levels and IgG3 hinge region phenotypes were used for the outcome of clinical malaria. Infants were screened monthly for clinical malaria and any other times when there

was rise in body temperature for 24 months (first 2 years of life). Infants were classified as non-protected from clinical malaria if they had malaria parasites and fever (Rogers *et al*, 2006), protected if they had asymptomatic parasitaemia (Males *et al*, 2008) of any parasite count and indeterminate if they had no parasites and no fever.

3.4 Sample Size Determination

The incidence of malaria parasitaemia in the Kintampo Birth Cohort Study was about 70% (Asante *et al*., 2013) as the likely incidence of clinical malaria. With a standard (z) score of 1.96 at 95% confidence level and 5% allowable error margin, the equation

$$n = \frac{\left(z_{1-\alpha}\sqrt{2\bar{P}(1-\bar{P})} + z_{1-\beta}\sqrt{P_0(1-P_0)+P_1(1-P_1)}\right)^2}{(P_1-P_0)^2}$$

where n-the minimum sample size, z-the standard score, p-the known prevalence of malaria, and e, the allowable error margin, the minimum number of samples required for the study is 103. The study was powered to obtain a 10% (absolute) margin of error (precision), and a design effect of 1.2. With these assumptions, EpiInfo version 7.0 was used to estimate the required sample size of 103 with an assumption of 10% error in the sample results, a minimum of 112 children were needed for the analyses. Thus a total of 202 children's plasma samples and 138 filter paper samples (blood blots) were taken for immunological and molecular analyses respectively by random sampling.

3.5 Inclusion criteria

Infants who had clinical malaria in the first year of life (early months) with evidence of fever and parasitaemia were included in the study. Also infants without clinical malaria with the evidence of no parasitaemia and without fever were included in this study.

3.6 Exclusion criteria

Infants with other illnesses apart from clinical malaria were excluded. Also infants with parasitaemia but having co-morbidities were excluded from this study since any clinical symptom may not easily be attributed to the presence of parasites or the co-morbidities.

3.7.0 Laboratory Evaluations/Assay

All the ELISAs were done in the immunology department of NMIMR under the supervision of the head of the serology laboratory and molecular (samples extraction and PCR) analyses conducted at molecular biology laboratory, KHRC under the supervision of the head of KHRC molecular laboratory.

3.7.1 Specimen Collection and Storage

Plasma samples from the Kintampo Birth Cohort Study were already stored at -80^0C. Plasma samples from the same study were also transported from Kintampo on dry-ice to

the immunology laboratory at NMIMR for analyses in Accra- Ghana. Blood blots had already been prepared from whole blood on a filter paper for genotyping.

3.7.2 Enzyme-Linked-Immunoassays

IgG and IgG subclass levels to the recombinant malarial antigens (GLURP R0 and MSP1-19) were measured by indirect ELISA using the Afro Immuno Assay (AIA) ELISA protocol described by Nebie and Lusingu (Nebie *et al*., 2008, Lusingu *et al*., 2005) with a little modification made in the concentrations of the coating antigens and secondary/detection antibodies. Prior to the ELISAs, plasma samples were diluted at 1:200 in plasma dilution buffer (PBS with 2.5 % milk powder, 0.1% Tween-20 and 0.02% Na-azide). At the end of the experiment, samples which had very low absorptions beyond detectable limits were re-diluted at 1:100 and 1:50 dilutions especially infants with low antibody concentrations. All dilutions were entered into a software (ADAMSEL40-version1.1) for antibody estimation.

Each well in the 96-well microtitre ELISA plate (Maxisorp Nunc, Denmark) was directly coated with 100µl of antigens in coating buffer (plain PBS, pH 7.04) at 1.0µg/ml. Coated plates were kept in a monitored refrigerator at 2°C to 8°C overnight (12 hours minimum incubation). The plates were washed four times with washing buffer (PBS with 0.1% Tween-20) using the Biotek ELx 405 automated ELISA plate washer (Biotek Instruments, Winooski, VT; USA). The washed plates were padded dry on a tissue paper and blocked with 200µl of blocking buffer (PBS with 5 % milkpowder, 0.1% Tween-20) and incubated at room temperature in a humidified chamber for 1 hour. Plates were washed four times with washing buffer, padded dry and diluted plasma samples (1:200 or 1:300) added at

100µl/well in duplicates. The plates with the diluted plasma samples were incubated at room temperature for 2 hours in a humidified chamber after which they were also washed four times using washing buffer and a secondary (detection) antibody for the specific antibody to be determined added at 100µl/well (The optimized dilutions for the detection antibodies used in the assays were; goat anti-human IgG (γ) horseradish peroxidase (HRPO) conjugated (Invitrogen Corporation, Camarillo, CA; USA) (1:80000) for the IgG isotype and the IgG subclasses were detected using HRPO conjugated sheep polyclonal (The Binding Site Group Ltd, Birmingham; UK) IgG1 (1:5000), IgG2 (1:2000), IgG3 (1:10000) and IgG4 (1:1000) antibodies respectively. For IgM, goat anti-human IgM (µ) HRPO conjugated (Invitrogen Corporation, Camarillo, CA; USA) (1:3000) for the isotype). The plates with the secondary antibodies conjugates were incubated for 1 hour at room temperature in a humidified chamber after which they were washed four times with washing buffer using the an automated plate washer and padded dry on a paper tissue. Bound secondary antibodies were quantified with ready to use TMB (3, 3', 5, 5'-Tetramethylbenzidine) substrate (Kem-En-Tec Diagnosis A/S, Taastrup, Denmark) which develops a yellow colour when incubated in the dark for 30 min. The intensity of the colour developed was directly proportional to antibody concentration. The optical density (OD) were read at 450 nm with Biotek EL 808 ELISA plate reader (Biotek Instruments, Winooski, VT; USA). Optical density values for the test samples were converted into antibody units (AU) with the standard reference curves generated for each ELISA plate using a four parameter curve-fit Microsoft Excel-based application (ADAMSEL b040, Ed Remark© 2009).

3.7.2.1 Quality Control for the ELISAs

To control for inter-assay and day-to-day variations in the standardized ELISA procedure, each assay (ELISA plate) had a calibration curve obtained by a 2-fold titration of pool of hyper immune sera known to be positive for the antibodies (total IgG, IgG1, IgG 2, IgG3, IgG4, IgM) to the specified antigens tested. Each plate also had a negative control sample (plain buffer solution), a positive control sample (plasma from a clinically immune adult obtained from the Korle-Bu blood bank, Accra) and a buffer blank (serum dilution buffer without serum sample) which served as internal controls to allow for detection of a failed assay run. The samples were run in duplicates and the mean calculated using Adamsel program.

3.7.3.0 Molecular Analysis

3.7.3.1 DNA Extraction

Archival blood blots on protein saver filter papers (Whatman 903 ^{TM} filter paper, USA) was used. Qiagen kit for DNA extraction and purification (Qiagen kit, USA) were used according to the manufacturer's instructions.

About 3mm diameter of blood blot from a filter paper was cut into labelled 1.5ml eppendorf tubes. 180 ul of ATL buffer from Qiagen was added and incubated at 85^{O}C on a heat Block with a thermostat and thermometer (grant instruments Cambridge LTD, England) for 10 min and centrifuge for 10 sec at 8000 rpm (Galaxy 14D). 20ul of proteinase K stock solution was added and vortexed (grant instruments Cambridge LTD, England) for 1min

to mix, incubated for 1hour in a water bath (Clifton, nickel electro limited, England) and mixture centrifuged for 10 min at 8000 rpm. 200 ul of AL buffer was added to the sample and mixed immediately by vortexing for about 1 minute. The mixture was incubated at 70^0C on a hot plate for 10 min and centrifuged at 800 rpm for 10 sec. 200 ul of absolute ethanol was added, mixed by vortexing for 2 min after which centrifugation was done at 8000 rpm for 10 sec. The mixture was carefully transferred into the QIAamp mini spin column (QMSC) (in a 2ml collection tube) using 1000 ul pipette, capped closed and centrifuge at 8000 rpm for 1min. The QMSC was placed in a clean 2ml collection tube and the tube containing the filtrate discarded. The QMSC was carefully opened and 500 ul AW1 buffer added into each tube and centrifugation done at 800rpm for 1 min. The QMSC was placed in another clean 2ml collection tube and the tube containing filtrate after centrifugation was discarded. The QMSC was opened and 500 ul of AW2 buffer added into each tube and centrifuged at 13000 rpm for 4 min. The QMSC was placed in a new 2ml collection tube (not provided with kits) and the collection tube discarded with the filtrate and centrifuged at 13000 rpm for 1min.

The QMSC was placed in labelled 1.5ml eppendorf tubes avoiding carrying over of AW2 buffer from previous steps. The tube containing filtrate was discarded. The QMSC was carefully opened and 100 ul of AE buffer was added and incubated at room temperature for 3 mins. Centrifugation was done at 8000 rpm for 1min. discarding the column and the eluate which contained the DNA stored immediately in -20^0C.

3.7.3.2 Genotyping of IgG3 Hinge Region Length Polymorphism by PCR

The IgG3 hinge region was amplified using, sense (5'–AAAACCCCACTTTGGTGACAC) and antisense (5'-GGGTCCGGGAAATCATAAGG) primers (Adu, 2010) (DNA Technology, A/S, Denmark) designed to anneal to specific sequences in exon 2 and exon 5 respectively to amplify the fragment encoding the hinge region of human IgG3 from genomic DNA. The PCR reaction mixture was made of 10-30 nanograms of genomic DNA, 10millimolar (mM) of primer (sense and antisense), 1.25mM of each of the dNTPs, 1 unit of HotStarTaq® DNA polymerase and the corresponding 10X HotStar reaction buffer in a total volume of 25ul. The PCR cycling conditions was an initial denaturation at 95^0C for 15 mins, followed by 38 cycles consisting of a denaturation step at 95^0C for 30 secs, an annealing step at 61^0C for 30 secs, an elongation step at 72^0C for 30 secs and then a single final elongation step at 72^0C for 7mins (Adu, 2010).

3.7.3.3 Analyses of the PCR product

After the PCRs 5µl of the PCR products were then separated on 2% agarose gel (SeaKem® GTG® Agarose, Lonza, Rockland, ME, USA) in 0.5X Tris-EDTA running buffer (Biopioneer Co, USA) by electrophoresis at 90volts (Apelex Power station, France) for 60 minutes using 1µl of blue DNA loading dye (Promega Co, USA) and stained with 0.5µg/ml ethidium bromide (Life Technologies Co, USA). Hundred base pair nucleotide sequence molecular size marker (Ladder IV) (Sigma Mo, USA) was run alongside the PCR products on the gel. The gel was visualized and pictures (electrophorogram) taken using UV-

illumination (AlphadigiDocTM, Alpha innotech corporation, EEC) and the pictures were then analyzed by comparing the text sample to the Ladder.

3.8 Statistical Analyses

Data was analyzed using STATA 11.0. The goal of this analysis was to identify relationships between antibody responses to specific *P. falciparum* antigens and clinical malaria outcome. Logistic regression models was used to assess the relationship between specific genotypes or polymorphism and the risk of clinical malaria. Logistic regression models were used to analyze for associations between antibody responses and categorical variables such as presence or absence of clinical malaria episodes during the period following each scheduled serum sampling.

3.9 Ethical Considerations

Mothers or caregivers were consented and enrolled in the Kintampo Birth Cohort Study. The Kintampo health research centre's - Institutional Ethics committee (KHRC-IEC) gave approval for the main birth cohort study and University of Ghana Medical School's Ethical Review Committee (UGMS-ERC) gave approval for the use of birth cohort study samples and clinical data for this study. Data on participants were not shared with anyone apart from study supervisors and samples were used for tests approved by the ethics committee.

CHAPTER FOUR

4.0 RESULTS

4.1 Clinical and demographic data of study subjects for antibody study.

A total of 202 archived plasma samples were selected for antibody analyses from a birth cohort study conducted in the Kintampo North Municipality, the Kintampo South Districts, and the Nkoranza South District.in Ghana. Among these 202 infants, 112 (55.45%) were not protected from clinical malaria (presence of parasites and fever), 68 (33.66%) had asymptomatic parasitaemia (protected) and 22 (10.89%) had no parasites and no fever (indeterminate group or non-exposed group).

Kintampo municipality is divided into rural areas which have deprived social amenities, potable water health facilities with more incidence of clinical malaria cases and the urban areas having better social amenities, potable water health facilities with less incidence of clinical malaria cases. Factors that could be confounding variables such as use of bed net, suburb, season of birth were captured and adjusted for in the statistical analyses.

From Table 4.1, the use of bed net, sex, and season of birth of infants influenced their susceptibility to clinical malaria ($p < 0.05$). Suburb had no effect on protection from clinical malaria in infants born to these mothers ($p > 0.05$l). These were confounders and were accounted for in the analyses.

Tables 4.1 Demographic data of participants protected and non-protected from clinical malaria (N=180)

Co-variate	Non-protected n (%)	Protected n (%)	Odds Ratio (95 CI)	P-value
Sex				
Male	62 (68.13)	29 (31.87)	1.199 (1.068-1.345)	0.002
Female	50 (56.18)	39 (43.82)		
Season				
Dry	21(45.65)	25 (54.35)	0.849 (0.762-0.947)	0.003
Wet	91 (67.91)	43 (32.09)		
PM* mother				
No	68 (61.82)	44 (62.86)		
Yes	42 (38.18)	26 (37.14)	0.866 (0.794-1.005)	0.155
Suburb				
Rural	104 (61.90)	64 (38.10)	0.857 (0.700-1.049)	0.134
Urban	8 (66.67)	4 (33.33)		
Bed net use				
Yes	54 (65.85)	28 (34.15)	0.807 (0.697-0.934)	0.004
No	57 (64.77)	31 (35.23)		

*PM mother- placental malaria mother.

4.2 Maternally transferred antibodies against GLURP R0 and MSP1-19 at birth.

The levels of maternally transferred anti-GLURP R0 and anti-MSP1-19 IgG and their subclasses were determined at month 0 from cord blood at birth (Figure 4.1 i and ii). The geometric means of maternally transferred anti-GLURP R0 IgG was significantly higher than that of anti-MSP1-19 IgG at birth ($p < 0.05$) whiles the subclasses were not statistically different although IgG3 levels were slightly higher compared to the rest (Figure 4.1 i and ii).

The levels of maternally transferred antibodies against GLURP R0 and MSP1-19 at birth were compared between infants who had clinical malaria (presence of parasites > 1000ul/blood and fever), those protected from clinical malaria (asymptomatic parasitaemia at any level) and the indeterminate group (absence of parasites and no fever) for IgG, IgG1, IgG2, IgG3 and IgG4 (Figure 4.1 i and ii). There was a significant difference in the geometric means of maternally transferred anti-GLURP R0 and anti-MSP1-19 IgG levels between infants protected from clinical malaria and those not protected from clinical malaria ($p<0.05$, Figure 4.1 i and ii, Table 4.2). In general, high anti-GLURP R0 IgG titres were observed compared to anti-MSP1-19 IgG at birth ($p<0.05$, Figure 4.1 i and ii). However, there were no significant differences in the levels of the IgG subclasses against GLURP R0 and MSP1-19 ($p>0.05$ in all subclasses, Figure 4.1 i and ii, Table 4.2). Also there were no statistical difference in anti-GLURP R0 and anti- MSP1-19 IgG and subclasses levels for both protected and indeterminate group ($p>0.05$ in all cases, Figure 4.1 i and ii).

Figure 4.1 (i) geometric means of antibody levels at birth *against GLURP R0 (ii)* geometric means of *antibody levels at birth against MSP1-19. (iii) anti- GLURP R0 IgG3 Levels at Month 0, 3 and 6 in malaria negative and positive infants. (iv) Anti-MSP1-19 IgG3 Levels at Month 0, 3 and 6 in malaria negative and positive infants.*

Keys:

A - Infants with presence of parasites and fever (non-protected from clinical malaria).

B - Infants with asymptomatic parasitaemia of any level (protected from clinical malaria)

C - Infants with no parasites by microscopy and no fever (indeterminate group).

Table 4.2 Associations of maternally transferred antibodies with clinical malaria protection

Antigen	Antibody	Odds Ratio	95% CI	P-value
GLURP R0 at Month 0	IgG	1.005	0.784 – 1.288	0.040
	IgG1	1.047	0.483 - 0.922	0.70
	IgG2	1.030	0.911 - 1.165	0.634
	IgG3	0.964	0.886 – 1.050	0.403
	IgG4	1.042	0.323 - 0.961	0.323
GLURP R0 at Month 3	IgG3	0.914	0.839 – 0.995	0.039
GLURP R0 at Month 6	IgG3	1.001	0.921 – 1.088	0.983
MSP1-19 at Month 0	IgG	1.097	1.009 - 1.192	0.030
	IgG1	1.058	0.974 - 1.148	0.180
	IgG2	1.161	0.987 – 0.367	0.072
	IgG3	1.052	0.963 – 1.149	0.259
	IgG4	1.068	0.963 – 1.184	0.212
MSP1-19 at Month 3	IgG3	1.036	0.961 – 1.117	0.360
MSP1-19 at Month 6	IgG3	0.954	0.871 – 1.045	0.313

4.3 IgG3 against GLURP R0 and MSP1-19 at month 0, 3 and 6 of infants.

The geometric means of IgG3 against GLURP R0 and MSP1-19 were compared between infants protected from clinical malaria, non-protected and the indeterminate group at months 0, 3 and 6 (Figure 4.1 iii and iv respectively). There was a significant decrease in IgG3 titres from month 0 to month 6 in malaria protected, non-protected and infants with

indeterminate malaria outcomes for GLURP R0 (p-value < 0.001, T-test). The difference was as a result of the sharp decline in IgG3 titres from month 0 to month 3 (p-value < 0.039, Tables 4.2). There was no significant decrease in IgG3 titres from month 0 to month 6 in malaria protected, non-protected and infants with indeterminate malaria outcomes for MSP1-19 (p-value > 0.05 in month 3 and 6, Tables 4.2). Only IgG3 titres were determined at months 3 and 6 because this study focused more on the IgG3 levels, the hinge region polymorphisms and their decay rate.

4.4 Socio-demographics of the study infants for IgG3 hinge region polymorphism study

One hundred and thirty-eight (138) infants' samples were randomly selected for the IgG3 hinge region length polymorphisms which were different from the infants sampled for antibody study but two of the samples could not be analyzed since DNA could not be extracted from those samples (Table 4.3). Factors that could be confounding variables such as use of bed net, suburb, season of birth were captured and adjusted for in the statistical analyses.

Every infant in the IgG3 hinge region length polymorphism (IgG3HRLP) study was exposed to the parasite at least once before the 24th month of life (Tables 4.4 and 4.5). Before the 12 months of infants' life, about 93.33% of the infants had been exposed to the malaria parasites and there was no association with IgG3HRLP (p>0.05, Table 4.4).

For the hinge region analyses, the same case definitions were used; infants not protected from clinical malaria (presence of parasites and fever), protected (had asymptomatic

parasitaemia) and infants with no parasites with no fever as indeterminate group. With the indeterminate group PCR would be the tool of choice to confirm whether the infants were truly unexposed to the parasite or has infections very low to be detected by light microscopy.

Table 4.3 Socio-demographic characteristics of infants involved in the IgG3 hinge region length polymorphisms study (N=129)

Co-variate	Protected n (%)	Non-protected n (%)	Odds Ratio (95% CI)	P-value
Sex				
Male	12 (16.67)	60 (83.33)	1.19 (1.06,1.34)	0.003
Female	6 (10.53)	51 (89.47)		
Season				
Dry	10 (14.09)	61 (85.92)	0.86 (0.77, 0.96)	0.006
Wet	8 (13.79)	50 (86.21)		
Suburb				
Rural	16 (13.79)	100 (86.21)	0.84 (0.69, 1.03)	0.089
Urban	2 (15.38)	11 (84.62)		
Bed net use				
Yes	11 (14.87)	63 (85.14)	0.81 (0.70, 0.94)	0.006
No	4 (7.27)	51 (94.12)		

4.5 IgG3 hinge region length polymorphism Distributions among Infants

DNA from infants' samples were genotyped for polymorphisms in the IgG3 hinge region. Polymorphisms in the IgG3 hinge region were classified as L, M and S for Long, Medium and Short alleles respectively (electrophorogram Figure 8.7, appendix II). The gene distribution was in Hardy-Weinberg's equilibrium ($p > 0.05$).

The M allele was the most dominant with allelic frequency of 0.56 followed by the L-allele with 0.44. The s-allele was very uncommon in this population. The homozygote medium (MM) polymorphism had the highest frequency of 53.33%, followed by the homozygote long (LL) polymorphism with a frequency of 42.22% (Table 4.4). The homozygote short SS and heterozygote long-medium (LM) polymorphisms were very uncommon among these infants. Three (2.17%) of the SS hinge genotype were found but not included in this analysis. Other polymorphisms like LS and MS were not found in this study.

Table 4.4 IgG3HRLP Distributions among Infants and clinical malaria before one year

Genotype	IND* n (%)	Non-protected n (%)	Total	P-value
LL	6 (66.67)	51 (40.48)	57 (42.22)	
LM	0 (0.00)	6 (4.76)	6 (4.44)	
MM	3 (33.33)	69 (54.76)	72 (53.33)	
Total	9 (100.00)	126 (100.00)	135 (100.00)	0.430

*indeterminate. Key *L-long hinge region allele, M-medium hinge region allele*

4.6 Infants' IgG3 hinge region polymorphism and malaria protection of after year one.

The risk of clinical malaria was determined among 135 infants from 12 months (year one) to 24 months. Forty five (45) infants had clinical malaria whiles 90 infants were indeterminate between month 12 and month 24. There was an association between infants' IgG3 hinge region length polymorphisms and the risk of malaria after one year of life using the Fisher's exact ($p<0.05$, Table 4.5).

Table 4.5 infants' IgG3HRLP and malaria protection after year one.

infants' polymorphisms	Non-protected n (%)	IND* n (%)	Total	**P-value
LL	21 (46.67)	36 (40.00)	57 (42.22)	0.022
LM	5 (11.11)	1 (1.11)	6 (4.44)	
MM	19 (40.43)	53 (58.89)	72 (53.33)	
Total	45 (100.00)	90 (100.00)	135 (100.00)	

*indeterminate **p-value Fisher's exact test, LL –homozygote long, MM – homozygote medium, LM –heterozygote genotype.

A time to event graph for clinical malaria (survival curve) was obtained for the IgG3HRLP for this study (Figure 4.2). There was an intersection of all the three graphs at month 5 and 7 on the on the survival curve (Kaplan-Meier graph), after which the graphs started to show more distinct patterns. The time to event graph showed lower probability of not getting clinical malaria among infants with the LM genotype and the MM genotype showed a

higher probability of not getting clinical malaria (Figure 4.2). The difference was not statistically significant though, and was confirmed with the hazard ratios of the genotypes (Table 4.6). The hazard ratios for the various hinge region genotypes were estimated from month 12 to the time of exit from the study (24 months). There was no significant difference in the protective potential of the various genotypes ($p > 0.05$ at 95% CI, Table 4.6). Infants with LM genotype had 0.76 times lower chances of having clinical malaria compared with infants with LL genotype whiles infants with the MM genotype had 0.54 times lower chances of having clinical malaria compared to infants with LL genotype.

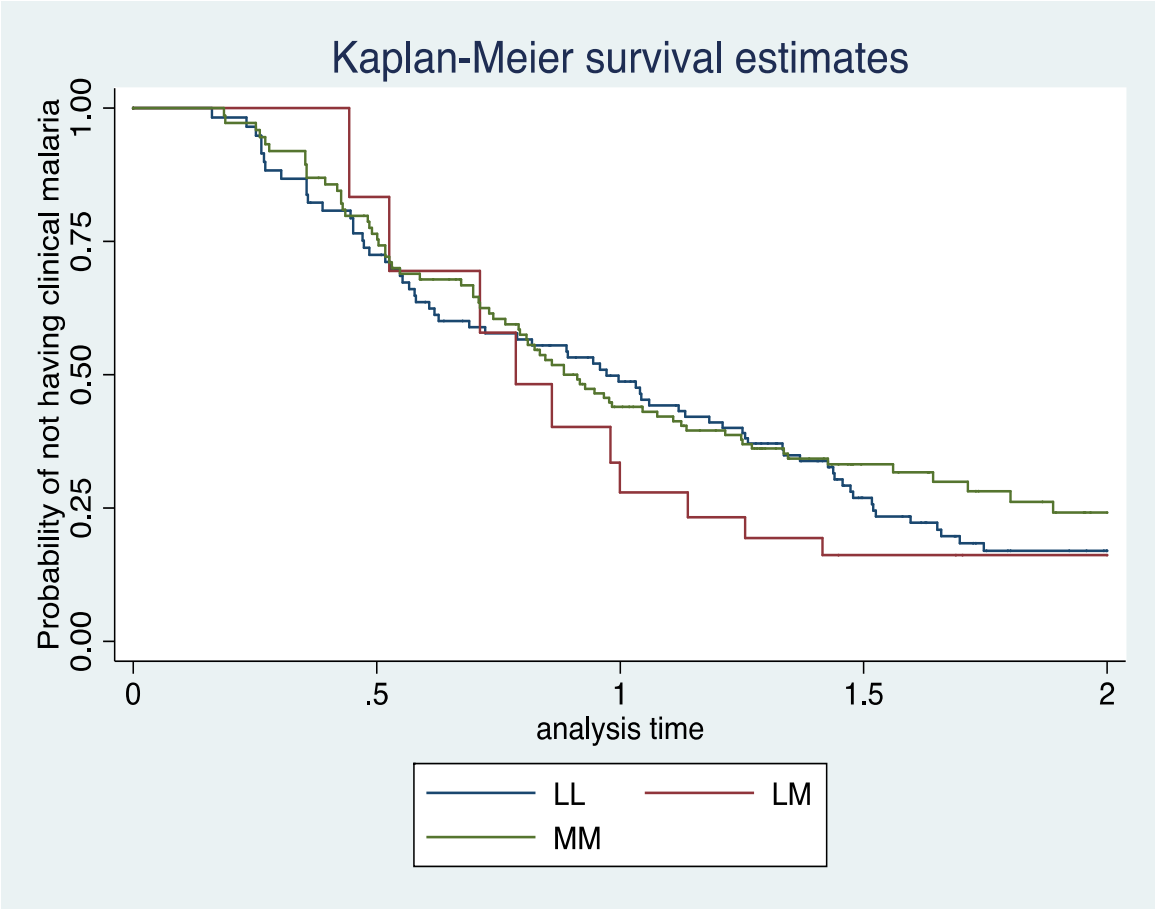


Figure 4.2 Time to clinical malaria graph for the various IgG3HRLPs. LL –homozygote long, MM – homozygote medium, LM –heterozygote genotype.

Table 4.6 Hazard ratios of the IgG3HRLP from 12 months to 24 months of infants' life.

Genotype	Hazard ratio	95% CI	*P-value
LL	1		
LM	0.76	0.25 - 2.36	0.636
MM	0.54	0.24 - 1.26	0.156

*p-values, hazard ratios and 95% CI were calculated by the Cox proportional hazards regression model. CI- confidence interval. LL –homozygote long, MM – homozygote medium, LM –heterozygote genotype.

CHAPTER FIVE

5.0 DISCUSSION

5.1 Anti-GLURP R0 and anti-MSP1-$_{19}$ antibody levels at birth and clinical malaria

Understanding humoral immunity to the *Plasmodium* parasite is key in vaccine development. The aim of this study was to determine maternally transferred antibodies and IgG3 hinge region length polymorphisms in the risk of clinical malaria in infants.

At the population level, there seem to be some evidence of an association between decreasing levels of maternally derived malaria-specific IgG and increasing risk of clinical malaria (Akanmori *et al*., 1995, Campbell *et al*., 1980). However, in a review on maternally transferred malaria-specific antibodies and infant malaria, it was concluded that there was lack of efficacy of passively acquired maternal antibody in neonates (Brabin, 1990). There are conflicting results from different studies in different settings (Riley *et al*., 2001).

In this study, there were high titres of maternally transferred anti-GLURP R0 IgG at birth compared to anti-MSP1-19 IgG in all the infants possibly reflecting the immunodominant nature of GLURP R0. However, higher titres of IgG against both antigens were associated with reduced risk of clinical malaria.

In agreement with other studies, the higher titres of maternally transferred antibodies at birth (anti-GLURP R0 IgG and anti- MSP1-$_{19}$ IgG) were associated with neonates protection from clinical malaria (Akanmori *et al*., 1995, Campbell *et al*., 1980). This study

however was in disagreement with one of the most comprehensive birth cohort study in the coastal Ghana that had no association with maternally transferred antibodies (Riley *et al*., 2000). The high IgG titres observed in protected infants in this study may indicate the boosting of maternal serum antibodies due to malaria parasite infection (Brabin, 1990) and may induce some level of protection or reduce the risk of clinical malaria in neonate if more titres are transferred. This was evident in the significantly higher anti-GLURP R0 and anti-MSP1-$_{19}$ IgG levels in infants protected from clinical malaria (had parasite but did not get sick/febrile) than infants not protected from clinical malaria (had parasites and got sick/febrile). The indeterminate group had an intermediate levels that was between the levels of the protected and non-protected infants. Some infants in the indeterminate group had quite higher total IgG levels which may reflect possible low grade infections that might have been skipped by microscopy, or too low to detect with microscopy (Rantala *et al*., 2010).

Epidemiological studies in older people have linked higher antibodies to GLURP R0 and MSP1-$_{19}$ with immunity to clinical malaria (Egan *et al*., 1996, Al-Yaman *et al*., 1996) and this protection was also shown by this study in newborn infants. Protection was not surprising with these high IgG titres because neonates have relatively matured phagocytes (Quie, 1990) to clear the parasite after the binding of the 'effective maternally acquired immunoglobulins to parasites. Neutrophil numbers in infants circulation are high in the normal neonate (not preterm babies) with relatively mature lymphocyte system and their mononuclear cells have normal antigen-presenting and secretory function (Quie, 1990) to eliminate antibody opsonized parasites. This may further be confirmed using maternally

transferred IgG and subclasses in infants, and infants' isolated peripheral blood mononuclear cells (PBMCs) in parasite growth inhibition assays at different weeks of life.

The differences in IgG subclasses titres were not significant in the protected, non-protected infants and the indeterminate group against the two antigens at birth. This may be that these maternally transferred IgG subclasses may be purposefully to neutralize parasites that may bind/cross the placenta. The antibodies may have some degree of protection/reducing risk of clinical malaria in infants in early post natal life. However, their synergistic effect was seen in the total IgG to the antigens.

Immunoglobin G3 among the subclasses had higher levels comparatively at birth though not statistically significant. The elevated level of maternally transferred anti-GLURP R0 and anti-MSP1-$_{19}$ IgG3 in infants may suggest that IgG3 crosses the placenta more easily compared to the other subclasses (Hay *et al.*, 1971). Their titres were not so different in both malaria protected and non-protected infants at birth.

5.2 The levels of IgG3 to GLURP R0 and MSP1-$_{19}$ at month 0, 3 and 6 of infants.

This study recorded a sharp decay of maternally transferred IgG3 against both antigens from day of delivery to the month 3 in both protected, non-protected and indeterminate group. This perhaps may be due to catabolism of maternally acquired Immunoglobulins (Hviid and Staalsoe, 2004) than the use of the immunoglobulin in fighting malaria infections. Other studies observed a similar pattern with maternally acquired antibodies to other infectious diseases (Sood *et al*., 1995, Leuridan *et al*., 2010) such as measles, mumps, rubella, and varicella with waning period of less than 3.3, 2.7, 3.9 and 3.4 months

respectively (Waaijenborg *et al*., 2013). It is the rapid waning of the antibodies that leaves infants vulnerable (Healy *et al*., 2004) until their own adaptive immunes system begins to mount responses.

The rise in anti-GLURP R0 and anti MSP1-19 IgG3 titres in non-protected infants from month 3 to 6 was due to infections that stimulated infants' production of antibodies (IgG3). However, protected infants had constant IgG3 titres possibly due to the unavailability of parasites to stimulate the immune system.

5.3 IgG3 hinge region length polymorphism and clinical malaria

Hosts' genetic factors play vital roles in immunity to certain infectious diseases making some individuals susceptible to infections while others may be protected. There has been growing evidence of ethnic differences in susceptibility to malaria and other genetic adaptations to malaria. Also there are epidemiological confirmation that Glucoses-6-Phospahte Dehydrogenase (G6PD) deficiency, α+ thalassemia, and hemoglobin C protect against malaria mortality and a growing number of reported associations with resistance and susceptibility to human malaria, particularly in genes involved in immunity, inflammation, and cell adhesion (Kwiatkowski, 2005).

The aim of this immunogenetic section was to determine the role of hinge region polymorphisms in IgG3 molecules in the outcome of clinical malaria in infants. The medium (M) allele and homozygote medium (MM) genotype for IgG3 hinge region length polymorphisms (IgG3HRLP) had the highest allelic and genotypic frequencies respectively in this study.IgG3 has been shown to be superior in parasite clearance

compared to the other IgG subclasses, a property which has been attributed to the fact that the hinge region of IgG3 is longer than hinge regions of the rest of the subclasses. The longer hinge allows for an increased flexibility and ability to link both antigens and FcγRs (Redpath *et al*., 1998, Roux *et al*., 1997). It was expected that this property of IgG3 should be more pronounced in polymorphisms with longer hinge region than those with medium and shorter hinge region, thus, making polymorphisms with longer hinge region more efficient at parasite clearance.

There was no association between IgG3 hinge region length polymorphisms (IgG3HRLP) in infants' malaria protection in the first year of life. This may possibly be due to the fact that from birth up to about the first six or nine months of infants' life, the maternally transferred antibodies play the most important role in protection from clinical malaria (Akanmori *et al*., 1995, Campbell *et al*., 1980) and in other infectious diseases (Puck *et al*., 1980). It is the rapid waning of the antibodies that leaves infants vulnerable (Healy *et al*., 2004) until the infants 'own adaptive immunes system begins to mount responses. The infants had not yet began to actively produce antibodies.

After one year of life however, there was an association in the infants' IgG3 hinge region length polymorphisms and clinical malaria using the student t-test. However, the Kaplan-Meier plot and the Hazard ratios showed no significant differences in the protective potentials among the various hinge region length polymorphisms. Notwithstanding the insignificance, more infants with LM hinge region genotype were susceptible to clinical malaria whiles the infants with MM genotype had reduced risk of clinical malaria.

Individuals with the MM phenotype are known to have high IgG3 titres whiles those with the LL phenotype have low IgG3 titres (Adu, 2010). The LL phenotype might possibly be degraded more rapidly by proteolytic enzymes. The influence of the hinge region on IgG3 titres was thought to affect immunity to clinical malaria (Adu, 2010).

This study and others found an association with season of birth of infants (Koram *et al*., 2000, Owusu-Agyei *et al*., 2002) and bed net use (Diallo *et al*., 2007) with protection of infants from malaria. However, suburb and been born to a woman with placenta malaria had no association with infant protection.

This finding may require extensive work in order to incorporate it as a possible confounding variable in vaccine trials since polymorphisms in the hinge region of IgG3 appears to influence protection from clinical malaria.

Extensive work may include using maternally transferred IgG3 with the various polymorphisms in parasite growth inhibition assays at different weeks of infants' life and after one year of life.

CHAPTER SIX

6.0 CONCLUSION AND RECOMMENDATION

6.1 Conclusion

The maternally transferred Immunoglobulins IgG against GLURP R0 and MSP1-19 contributed to the protection of infants from clinical malaria. The IgG subclasses levels were not different among the protected, non-protected and indeterminate infants. There was a rapid waning of maternally transferred IgG3 levels by month 3 which may have resulted in more clinical malaria cases. Infants' IgG3 hinge region length polymorphisms played no significant in protective against clinical malaria.

6.2 Recommendations

It would be good to follow up with the IgG and subclasses for month 12, 18 and 24 to see the trend of rise and malaria incidence. It is also important to conduct this study using progressive malaria incidence to confirm protection from malaria.

Using maternally transferred IgG3 with the various polymorphisms (by conducting IgG3HRLP on maternal DNA samples) and infants' isolated PBMCs/plasma in parasite growth inhibition assays at different weeks of life and after one year of life is recommended.

In subsequent studies, the IgG3 levels and the IgG3HRLP should be conducted on the same participants in order to be able to compare the effect of the IgG3HRLPs on the IgG3 levels and waning. This was a limitation to this study.

Molecular techniques (PCR) should be used to confirm parasitaemia and unexposed infants among the indeterminate group in subsequent work.

7.0 APPENDIX I

7.1 Preparation of plain Phosphate buffered saline (PBS) solution

One tablet of Phosphate buffered saline was added to a 500ml double distilled water (ddH_2O) in a conical flask and place on a magnetic stirrer device. The magnetic stirrer was dropped into the flask and the device switched on. The magnetic stirrer stirs the ddH_2O with the PBS tablet to dissolve and PH tested with a pH meter.

7.2 Preparation of blocking buffer (PBS with 5 % milk powder, 0.1% Tween-20)

A 100ml PBS was measured and 5g of 1% fat milk powder weighed on a chemical balance into the PBS. Magnetic stirrer was dropped in the PBS with milk powder and then placed on a magnetic stirrer device to stir into a homogenous solution. 1ml of the solution was pipetted out and replaced with 1ml of the Tween-20 and stirring continued for about a minute.

7.3 Preparation of humidified chamber

A squared glass chamber (25cm*25cm, 5cm in height) was taken and a clean paper tissue was soaked in a ddH_2O and placed in the chamber with lid to create humidity.

7.4 Plasma samples diluted (PBS with 5 % milk powder, azide)

A 1000ml PBS solution was measured and 50g of 1% fat milk powder weighed on a chemical balance into the PBS. A magnetic stirrer was dropped in the PBS with milk powder and then placed on a magnetic stirrer device to stir into a homogenous solution. 10ml of the milk solution was pipetted out and 10ml of azide added and stirred on the magnetic stirrer device with the magnetic stirrer. 5ml (5000ul) of the solution was pipetted into 15ml falcon tubes labelled with the IDs of the samples to be analyzed:

For 1:200 samples dilution, 25ul of the solution was pipetted out of the solution in the falcon tubes and replaced with 25ul of plasma samples and vortexed for 30sec.

For 1:100 samples dilution, 50ul of the solution was pipetted out of the solution in the falcon tubes and replaced with 50ul of plasma samples and vortexed for 30sec.

7.5 Washing Buffer (PBS with 0.1% Tween-20)

In preparing 1000ml washing buffer, 2 tablets of PBS were added to a beaker containing 1000ml ddH_2O and placed on a magnetic stirrer without heating and stirred until all is in solution and 5.0ml of Tween-20 added. The solution was stirred until all had dissolved.

7.6 Color Solution [TMB (3, 3', 5, 5'-Tetramethylbenzidine)]

A ready to use substrate solution, TMB (3, 3', 5, 5'-Tetramethylbenzidine) was obtained from manufacturer (Kem-En-Tec Diagnosis A/S, Taastrup, Denmark) and added to plates at 100µl/well without any dilutions.

7.7 Stop Solution (0.2M H_2SO_4)

In preparing 1000ml of stop solution, 20.0ml of 10.0M H_2SO_4 was added to 980.0ml of ddH2O and the solution shaken to mix homogeneously. It was then cooled to room temperature and kept in the hood.

8.0 APPENDIX II

8.1 PCR Buffers

HotStar buffer and Nuclease free water were supplied by manufacturer (Amplicon, Hamburg, Germany) and used directly.

8.2 Primers for PCR

The primers [Sequences from Theisen ***et al.*** IGHG3-H1.1-F: 5' - AAA ACC CCA CTT TGG TGA CAC and IGHG3-CH2.1R: 5' - GGG TCC GGG AAA TCA TAA GG] used in PCR reaction were already reconstituted primers from manufacturer (DNA Technology A/S, Risskov, Denmark) which were diluted to working concentrations using PCR grade water.

8.3 5X Gel Loading Buffer

Gel loading buffer was supplied by manufacturer (Amplicon, Hamburg, Germany) and used directly. It was stored at room temperature.

8.4 1X Tris Acetate (TAE) Buffer

Stock concentrations of TAE buffer (C1) - 50X

Volume of the stock concentration used in making the working concentration (V1) - ?

Working concentration (C2) - 1X

Volume of working concentration need = 500ml

$C1 \times V1 = C2 \times V2$

$V1 = \frac{C2 \times V2}{C1}$

$V1 = \frac{1 \times 500ml}{50}$

V1= 10ml

10ml of the stock 50x at room temperature was taken into a 500ml volumetric flak and then topped up to the 500ml mark with distilled water, the resultant solution was shaken to mix and stored at room temperature for future use.

8.5 Two percent (2%) agarose gel preparation and casting

Two grams of agarose was weighed using a digital balance into a heat resistant bottle and then 100ml of 1x TAE was added. The solution was missed and microwave to dissolve the agarose in the 1x TAE buffer. After allowing to solution to cool down to just above room temperature, 5µl of ethidium bromide was added and then mix. The resultant solution was poured into a gel casting stray and with comb inserted to create wells. On completion of the PCR, the products were electrophoresed on a 2% agarose gel and stained with 0.5µg/ml ethidium bromide to detect the presence of amplified DNA fragments. Five microliters of

each sample was added to 1µl of orange G (5X) gel loading dye for the electrophoresis. Hundred base pair (ladder IV) DNA molecular size marker (Sigma, MO, USA) was run alongside the PCR products. The gel was prepared and electrophoresed in 1X TAE buffer using a mini gel system at 100 volts for one hour and the gel photographed over a UV trans-illuminator.

8.6.0 Molecular Lab Analyses Protocols

8.6.1 IgG3 Hinge Region PCR Protocol

Reagent	**X 1 Reaction (µl)**	**X n Reactions**
IGHG3-H1.1-Forward Primer (20mM)	0.5 µl	
IGHG3-CH2.1-Reverse Primer (20mM)	0.5 µl	
dNTP Mix (made of 10mM of each dNTP)	0.5 µl	
10X HotStar Buffer	2.5 µl	
HotStar DNA Polymerase	0.2 µl	
Water	20.3 µl	
Genomic DNA Template	0.5 µl (20 – 40 ng/µl)	
Total Volume	25.0 µl	

8.6.2 PCR Program

	Temp (°C)	Time	Temp (°C)	Time	Temp (°C)	Time	Cycle(s)
1	95.0	15 mins					1
2	95.0	30 sec	61.0	30 sec	72.0	30 sec	38
3	72.0	7 mins					
4	10.0	∞					

Primer Sequences ***(Theisen et al.)***

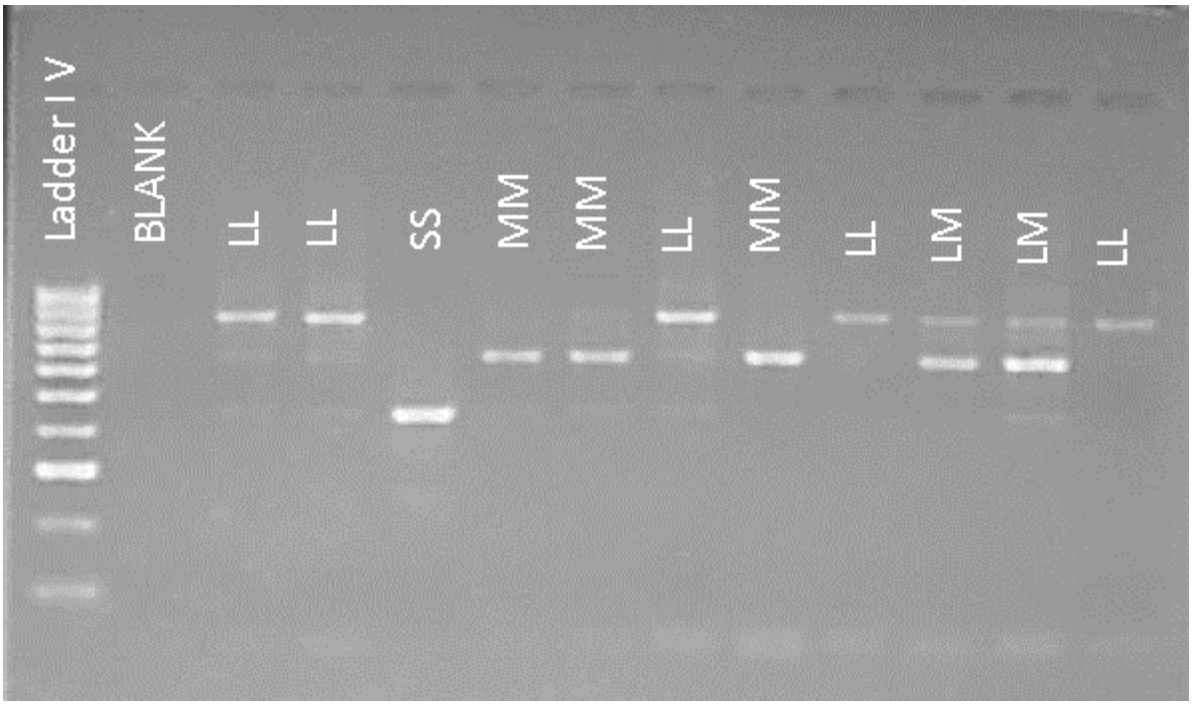
Forward primer

IGHG3-H1.1-F: 5' - AAA ACC CCA CTT TGG TGA CAC

Reverse primer

IGHG3-CH2.1R: 5' - GGG TCC GGG AAA TCA TAA GG

Figure 8.7 An electrophorogram of the PCR product



The ladder IV is a 100bp marker used as a point of reference to the gene band size. Blank contained no DNA samples but was treated as test sample to ascertain the quality of work in respect to contamination. The L genotype was between 800-900bp but was almost on the 800bp. The M genotype was between 600-700bp but closer to the 600bp mark and the S genotype between 400-500bp and closer to the 400bp mark. The genes were not sequenced in this work however, Adu *et al.* stated that the L, M and S genotypes were 807bp, 611bp and 423bp respectively according to their sequencing. LL indicated homozygote long, SS homozygote short, MM homozygote medium and LM indicated heterozygote long-medium.

8.7 Material Used

1. 10x PCR buffer
2. 1M HCl
3. 70% ethanol
4. Aerosol Filter Pipette Tips
5. Agarose
6. Electrophoresis set up (Labnet International, Power station 300)
7. Ethedium Bromide
8. Gel photography system (UVIsave gel documentation system, model GAS9200/1/2/3, Version 12)
9. $MgCl_2$
10. Micro-centrifuge
11. Micro-centrifuge tube
12. Nuclease free water
13. PCR Machine (Techgene)
14. Pipette
15. Taq polymerase
16. The automated H and E staining system (Leica Auto Stainer XL)

Hundred base pair of DNA molecular size markers

9.0 REFERENCE

ABBAS, A. K., MURPHY, K. M. & SHER, A. 1996. Functional diversity of helper T lymphocytes. *Nature,* 383**,** 787-793.

ABDALLA, S., WEATHERALL, D., WICKRAMASINGHE, S. & HUGHES, M. 1980. The anaemia of P. falciparum malaria. *British journal of haematology,* 46**,** 171-183.

ADU, B. 2010. *Immunological and Genetic Correlates of Immunity to Plasmodium Falciparum Malaria.* DEPARTMENT OF BIOCHEMISTRY AND BIOTECHNOLOGY, KWAME NKRUMAH UNIVERSITY OF SCIENCE AND TECHNOLOGY, KUMASI.

AFUDEGO, C. E. 2012. COST EFFECTIVENESS ANALYSIS OF INSECTICIDE TREATED MOSQUITO NETS (ITNs) AND INDOOR RESIDUAL SPRAYING (IRS). *MALARIA INTERVENTIONS IN GHANA.* Accra: Ghana Health Service-Ministry of Health.

AKANMORI, B., AFARI, E., SAKATOKU, H. & NKRUMAH, F. 1995. A longitudinal study of malaria infection, morbidity and antibody titres in infants of a rural community in Ghana. *Transactions of the Royal Society of Tropical Medicine and Hygiene,* 89**,** 560-561.

AKAZILI, J., AIKINS, M. & BINKA, F. N. 2008. Malaria treatment in Northern Ghana: What is the treatment cost per case to households? *African Journal of Health Sciences,* 14**,** 70-79.

AL-YAMAN, F., GENTON, B. & KRAMER, K. 1996 Assessment of the role of naturally acquired antibody levels to Plasmodium falciparum merozoite surface protein-1 in

protecting Papua New Guinean children from malaria morbidity. . *American Journal of Tropical Medicine and Hygiene* 54**,** 443–448.

AREGAWI, M., CIBULSKIS, R. E., OTTEN, M. & WILLIAMS, R. 2009. *World malaria report 2009*, World Health Organization.

ARTAVANIS-TSAKONAS, K., TONGREN, J. & RILEY, E. 2003 The war between the malaria parasite and the immune system: immunity, immunoregulation and immunopathology. *Clinical & Experimental Immunology* 133**,** 145-52.

ASANTE, K. P., OWUSU-AGYEI, S., CAIRNS, M., DODOO, D., BOAMAH, E. A., GYASI, R., ADJEI, G., GYAN, B., AGYEMAN-BUDU, A. & DODOO, T. 2013. Placental malaria and the risk of malaria in infants in a high malaria transmission area in Ghana: a prospective cohort study. *Journal of Infectious Diseases,* 208**,** 1504-1513.

BALLOU, R. W. 2007. Obstacles to the development of a safe and effective attenuated pre-erythrocytic stage malaria vaccine. *Microbes and infection,* 9**,** 761-766.

BEAVIL, A. J., YOUNG, R. J. & SUTTON, B. J. 1995. Bent domain structure of recombinant human IgE-Fc in solution by x-ray and neutron scattering in conjunction with an automated curve fitting procedure. *Biochemistry***,** 34:14449.

BELNOUE, E., KAYIBANDA, M., VIGARIO, A. M., DESCHEMIN, J.-C., VAN ROOIJEN, N., VIGUIER, M., SNOUNOU, G. & RÉNIA, L. 2002. On the pathogenic role of brain-sequestered αβ CD8+ T cells in experimental cerebral malaria. *The Journal of Immunology,* 169**,** 6369-6375.

BRABIN, B. 1990. An analysis of malaria parasite rates in infants: 40 years after Macdonald. *Tropical Diseases Bulletin,* 87**,** 1-21.

BRAY, R. & GARNHAM, P. 1982. The life-cycle of primate malaria parasites. *British medical bulletin,* 38**,** 117-122.

BREMAN, J., ALILIO, M., WHITE, N. J., NOSTEN, F. & WHITE, N. J. 2007. Artemisinin-based combination treatment of falciparum malaria. *The American journal of tropical medicine and hygiene,* 77**,** 181-192.

BUCKLING, A. & READ, A. F. 2001. The effect of partial host immunity on the transmission of malaria parasites. *Proceedings of the Royal Society of London. Series B: Biological Sciences,* 268**,** 2325-2330.

BURTON, D. R. & WOOF, J. M. 1992. Human antibody effector function. *Advances in immunology,* 51**,** 1-84.

CAMPBELL, C. C., MARTINEZ, J. M. & COLLINS, W. E. 1980. Seroepidemiological studies of malaria in pregnant women and newborns from coastal El Salvador. *The American journal of tropical medicine and hygiene,* 29**,** 151-157.

CELADA, A., CRUCHAUD, A. & PERRIN, L. H. 1983. Assessment of immune phagocytosis of Plasmodium falciparum infected red blood cells by human monocytes and polymorphonuclear leukocytes. A method for visualizing infected red blood cells ingested by phagocytes. *Journal of immunological methods,* 63**,** 263-271.

CENTRE FOR DISEASE CONTROL 1999 MMWR supplement. 48(SU01)**,** 16-22.

CHOTIVANICH, K., UDOMSANGPETCH, R., SIMPSON, J. A., NEWTON, P., PUKRITTAYAKAMEE, S., LOOAREESUWAN, S. & WHITE, N. J. 2000. Parasite multiplication potential and the severity of falciparum malaria. *Journal of Infectious Diseases,* 181**,** 1206-1209.

COHEN, S., MCGREGOR, I. A. & CARRINGTON, S. 1961. Gamma-globulin and acquired immunity to human malaria. *Nature medicine,* 192**,** 733-737.

COICO, R. & SUNSHINE, G. 2009. *Immunology: a short course,* John Wiley & Sons.

COX-SINGH, J., DAVIS, T. M. E., LEE, K. S., SHAMSUL, S. S. G., MATUSOP, A., RATNAM, S., RAHMAN, H. A., CONWAY, D. J. & SINGH B. 2008. Plasmodium knowlesi malaria in humans is widely distributed and potentially life-threatening. *Clinical Infectious Diseases,* 46**,** 165–171.

DANGL, J. L., WENSEL, T. G., MORRISON, S. L., STRYER, L., HERZENBERG, L. A. & OI, V. T. 1988. Segmental flexibility and complement fixation of genetically engineered chimeric human, rabbit and mouse antibodies. *The European Molecular Biology Organization journal,* 7**,** 1989.

DARD, P., HUCK, S., FRIPPIAT, J. P., LEFRANC, G., LANGANEY, A., LEFRANC, M. P. & SANCHEZ-MAZAS, A. 1996. The IGHG3 gene shows a structural polymorphism characterized by different hinge lengths: sequence of a new 2-exon hinge gene. *Human genetics,* 99**,** 138-141.

DAVIES, D. R. & METZGER, H. 1983. Structural basis of antibody function. *Annual review of immunology,* 1**,** 87-115.

DAVIS, T., KARUNAJEEWA, H. A. & ILETT, K. F. 2005. Artemisinin-based combination therapies for uncomplicated malaria. *Medical Journal of Australia,* 182**,** 181-185.

DIAGNE, N., ROGIER, C., SOKHNA, C., TALL, A., FONTENILLE, D., ROUSSILHON, C., SPIEGEL, A. & TRAPE, J. 2000. Increased susceptibility to

malaria during the early postpartum period. *New England Journal of Medicine,* 343**,** 598-603.

DIALLO, D. A., SUTHERLAND, C., NEBIÉ, I., KONATÉ, A. T., ORD, R., ILBOUDO-SANOGO, E., GREENWOOD, B. M. & COUSENS, S. N. 2007. Children in Burkina Faso who are protected by insecticide-treated materials are able to clear drug-resistant parasites better than unprotected children. *Journal of Infectious Diseases,* 196**,** 138-144.

DODOO, D., ATUGUBA, F., BOSOMPRAH, S., ANSAH, N. A., ANSAH, P., LAMPTEY, H., EGYIR, B., ODURO, A. R., GYAN, B., HODGSON, A. & KORAM, K. A. 2011. Antibody levels to multiple malaria vaccine candidate antigens in relation to clinical malaria episodes in children in the Kasena-Nankana district of Northern Ghana. *Malaria Journal,* 10**,** 108.

DODOO, D., THEANDER, T. G., KURTZHALS, J. A., KORAM, K., RILEY, E., AKANMORI, B. D., NKRUMAH, F. K. & HVIID, L. 1999. Levels of antibody to conserved parts of Plasmodium falciparum merozoite surface protein 1 in Ghanaian children are not associated with protection from clinical malaria. *Infection and immunity,* 67**,** 2131-2137.

DURAISINGH, M. T., VOSS, T. S., MARTY, A. J., DUFFY, M. F., GOOD, R. T., THOMPSON, J. K., FREITAS-JUNIOR, L. H., SCHERF, A., CRABB, B. S. & COWMAN, A. F. 2005. Heterochromatin Silencing and Locus Repositioning Linked to Regulation of Virulence Genes in Plasmodium falciparum. *Cell,* 121**,** 13-24.

EGAN, A. F., MORRIS, J. & BARNISH, G. 1996. Clinical immunity to Plasmodium falciparum malaria is associated with serum antibodies to the 19kDa C-terminal fragment of the merozoite surface antigen, PfMSP1. *Journal of Infectious Diseases* 173**,** 765–769.

ELIADES, M. J., SHAH, S., NGUYEN-DINH, P., NEWMAN, R. D., BARBER, A. M., NGUYEN-DINH, P., ROBERTS, J., MALI, S., PARISE, M. & BARBER, A. 2005. Malaria surveillance—United States, 2003. *MMWR Surveill Summ,* 54**,** 25-40.

ENDO, S. & ARATA, Y. 1985. Proton nuclear magnetic resonance study of human Igs G1 and their proteolytic fragments: structure of the hinge region and effects of a hinge-region deletion on internal flexibilit. *Biochemistry* 24**,** 1561.

FIEVET, N., COT, M., CHOUGNET, C., MAUBERT, B., BICKII, J., DUBOIS, B., LEHESRAN, J., FROBER, T. Y., MIGOT, F. & ROMAIN, F. 1995. Malaria and pregnancy in Cameroonian primigravidae – humoral and cellular immune responses to Plasmodium falciparum blood-stage antigens. *American Journal of Tropical Medicine and Hygiene,* 53**,** 612-7.

FIEVET, N., TAMI, G., MAUBERT, B., MOUSSA, M., SHAW, I., COT, M., HOLDER, A., CHAOUAT, G. & DELORON, P. 2002. Cellular immune response to Plasmodium falciparum after pregnancy is related to previous placental infection and parity. *Malaria Journal* 16**,** 1-16.

FOWKES, F. J., RICHARDS, J. S., SIMPSON, J. A. & BEESON, J. G. 2010. The relationship between anti-merozoite antibodies and incidence of Plasmodium

falciparum malaria: A systematic review and meta-analysis. *PLoS medicine,* 7**,** e1000218.

FUNDENBERG, H., STITES, D. P., CALDWELL, J. L. & WELLS, J. 1976. *Basic and clinical immunology*, Lange Medical Publications, Los Altos, California 94022, USA.

GARDNER, M. J., HALL, N., FUNG, E., WHITE, O., BERRIMAN, M., HYMAN, R. W., CARLTON, J. M., PAIN, A., NELSON, K. E. & BOWMAN, S. 2002. Genome sequence of the human malaria parasite Plasmodium falciparum. *Nature,* 419**,** 498-511.

GRADY, C. Host defense mechanisms: An overview. Seminars in oncology nursing, 1988. Elsevier, 86-94.

GUPTA, A. P., CHIN, W. H., ZHU, L., MOK, S., LUAH, Y.-H., LIM, E.-H. & BOZDECH, Z. 2013. Dynamic Epigenetic Regulation of Gene Expression during the Life Cycle of Malaria Parasite Plasmodium falciparum. *PLoS pathogens,* 9**,** e1003170.

HANSEN, D. S., EVANS, K. J., D'OMBRAIN, M. C., BERNARD, N. J., SEXTON, A. C., BUCKINGHAM, L., SCALZO, A. A. & SCHOFIELD, L. 2005. The natural killer complex regulates severe malarial pathogenesis and influences acquired immune responses to Plasmodium berghei ANKA. *Infection and immunity,* 73**,** 2288-2297.

HARLOW, E. & LANE, D. 1988. *Antibodies: a laboratory manual*, Cold Spring Harbor Laboratory Cold Spring Harbor, NY.

HAY, F., HULL, M. & TORRIGIANI, G. 1971. The transfer of human IgG subclasses from mother to foetus. *Clinical and experimental immunology,* 9**,** 355.

HEALY, C. M., MUNOZ, F. M., RENCH, M. A., HALASA, N., EDWARDS, K. M. & BAKER, C. J. 2004. Prevalence of pertussis antibodies in maternal delivery, cord, and infant serum. *Journal of Infectious Diseases,* 190**,** 335-340.

HERMSEN, C. C., VERHAGE, D. F., TELGT, D. S., TEELEN, K., BOUSEMA, J. T., ROESTENBERG, M., BOLAD, A., BERZINS, K., CORRADIN, G. & LEROY, O. 2007. Glutamate-rich protein (GLURP) induces antibodies that inhibit in vitro growth of Plasmodium falciparum in a phase 1 malaria vaccine trial. *Vaccine,* 25**,** 2930-2940.

HILL, A. V., ALLSOPP, C. E., KWIATKOWSKI, D., ANSTEY, N. M., TWUMASI, P., ROWE, P. A., BENNETT, S., BREWSTER, D., MCMICHAEL, A. J. & GREENWOOD, B. M. 1991. Common West African HLA antigens are associated with protection from severe malaria. *Nature,* 352**,** 595-600.

HISAEDA, H., MAEKAWA, Y., IWAKAWA, D., OKADA, H., HIMENO, K., KISHIHARA, K., TSUKUMO, S.-I. & YASUTOMO, K. 2004. Escape of malaria parasites from host immunity requires CD4+ CD25+ regulatory T cells. *Nature medicine,* 10**,** 29-30.

HISAEDA, H., YASUTOMO, K. & HIMENO, K. 2005. Malaria: immune evasion by parasites. *The international journal of biochemistry & cell biology,* 37**,** 700-706.

HØGH, B., MARBIAH, N. T., BURGHAUS, P. A. & ANDERSEN, P. K. 1995. Relationship between maternally derived anti-Plasmodium falciparum antibodies

and risk of infection and disease in infants living in an area of Liberia, west Africa, in which malaria is highly endemic. *Infection and immunity,* 63**,** 4034-4038.

HOLDER, A. 1996. Malaria Vaccine Development: a Multi-Immune Response Approach ed. Hoffman, S.L., Washington, DC, . *American Society for Microbiology***,** 77–104.

HUCK, S., LEFRANC, G. & LEFRANC, M.-P. 1989. A human immunoglobulinIGHG3 allele (Gmb0, b1, c3, c5, u) with anIGHG4 converted region and three hinge exons. *Immunogenetics,* 30**,** 250-257.

HVIID, L. 2007. Development of vaccines against Plasmodium falciparum malaria: taking lessons from naturally acquired protective immunity. *Microbes and infection,* 9**,** 772-776.

HVIID, L. & STAALSOE, T. 2004. Malaria immunity in infants: a special case of a general phenomenon? *Trends in parasitology,* 20**,** 66-72.

JANEWAY, C. A., TRAVERS, P. & WALPORT, M. 2001. *Immunobiology: the immune system in health and disease,* New York, NY; Current Biology Publications, Churchill Livingstone.

JELINEK, T., GROBUSCH, M., SCHWENKE, S., STEIDL, S., VON SONNENBURG, F., NOTHDURFT, H., KLEIN, E. & LÖSCHER, T. 1999. Sensitivity and specificity of dipstick tests for rapid diagnosis of malaria in nonimmune travelers. *Journal of clinical microbiology,* 37**,** 721-723.

JONES, M. K. & GOOD, M. F. 2006. Life cycle of malaria infection. *Nature Medicine.,* 12**,** 170–171.

KANOI, B. N. & EGWANG, T. G. 2007. New concepts in vaccine development in malaria. *Current opinion in infectious diseases,* 20**,** 311-316.

KERSEY, J. H., SPECTOR, B. D. & GOOD, R. A. 1973. Primary immunodeficiency diseases and cancer: The immunodeficiency-cancer registry. *International Journal of Cancer,* 12**,** 333-347.

KORAM, K. A., OWUSU-AGYEI, S., UTZ, G., BINKA, F. N., BAIRD, J. K., HOFFMAN, S. L. & NKRUMAH, F. K. 2000. Severe anemia in young children after high and low malaria transmission seasons in the Kassena-Nankana district of northern Ghana. *The American journal of tropical medicine and hygiene,* 62**,** 670-674.

KRACKER, S. & RADBRUCH, A. 2004. Immunoglobulin Class Switching. *B Cell Protocols.* Springer.

KWIATKOWSKI, D. P. 2005. How malaria has affected the human genome and what human genetics can teach us about malaria. *The American Journal of Human Genetics,* 77**,** 171-192.

LAMBROS, C. & VANDERBERG, J. P. 1979. Synchronization of Plasmodium falciparum erythrocytic stages in culture. *The Journal of parasitology,* 65**,** 418-420.

LEURIDAN, E., HENS, N., HUTSE, V., IEVEN, M., AERTS, M. & VAN DAMME, P. 2010. Early waning of maternal measles antibodies in era of measles elimination: longitudinal study. *British Medical Journal,* 340.

LEUSEN, J. H. & NIMMERJAHN, F. 2013. The Role of IgG in Immune Responses. *Molecular and Cellular Mechanisms of Antibody Activity.* Springer.

LUSINGU, J. P., VESTERGAARD, L. S., ALIFRANGIS, M., MMBANDO, B. P., THEISEN, M., KITUA, A. Y., LEMNGE, M. M. & THEANDER, T. G. 2005. Cytophilic antibodies to Plasmodium falciparum Glutamate Rich Protein are

associated with malaria protection in an area of holoendemic transmission. *Malaria Journal* 4**,** 1-8.

MACPHERSON, A. J. & SLACK, E. 2007. The functional interactions of commensal bacteria with intestinal secretory IgA. *Current opinion in gastroenterology,* 23**,** 673-678.

MALES, S., GAYE O., & GARCIA, A. 2008. Long-term asymptomatic carriage of *Plasmodium falciparum* protects from malaria attacks, a prospective study among Senegalese children. *Clinical Infectious Diseases,* 46,516-22.

MARTENS, P., KOVATS, R., NIJHOF, S., DE VRIES, P., LIVERMORE, M., BRADLEY, D., COX, J. & MCMICHAEL, A. 1999. Climate change and future populations at risk of malaria. *Global Environmental Change,* 9**,** 89-107.

MAUBERT, B., FIEVE, T. N., TAMI, G., BOUDIN, C. & DELORON, P. 2000. Cytoadherence of Plasmodium falciparum-infected erythrocytes in the human placenta. *Parasite immunology,* 22**,** 191-9.

MCGREGOR, I. A., WILSON, M. & BILLEWICZ, W. 1983. Malaria infection of the placenta in The Gambia, West Africa; its incidence and relationship to stillbirth, birthweight and placental weight. *Transactions of the Royal Society of Tropical Medicine and Hygiene,* 77**,** 232-244.

MEEUSEN, E., BISCHOF, R. & LEE, C. 2001. Comparative T-cell responses during pregnancy in large animals and humans. *American Journal of Reproductive Immunology,* 46**,** 169-79.

MEIS, J., VERHAVE, J., JAP, P., SINDEN, R. & MEUWISSEN, J. T. 1983. Malaria parasites—discovery of the early liver form. 424-426.

MICHAELSEN, T., FRANGIONE, B. & FRANKLIN, E. 1977. Primary structure of the" hinge" region of human IgG3. Probable quadruplication of a 15-amino acid residue basic unit. *Journal of Biological Chemistry*, 252**,** 883-889.

MICHAELSEN, T. E., AASE, A., WESTBY, C. & SANDLIE, I. 1990. Enhancement of complement activation and cytolysis of human IgG3 by deletion of hinge exons. *Scandinavian Journal of Immunology***,** 32:517.

MILLER, J. L., SACK, B. K., BALDWIN, M., VAUGHAN, A. M. & KAPPE, S. H. 2014. Interferon-Mediated Innate Immune Responses against Malaria Parasite Liver Stages. *Cell reports*, 7**,** 436-447.

MILLINGTON, O., DI-LORENZO, C., PHILLIPS, R., GARSIDE, P. & BREWER, J. 2006. Suppression of adaptive immunity to heterologous antigens during Plasmodium infection through hemozoin-induced failure of dendritic cell function. *Journal of Biology*, 5**,** 172-9.

MINA-OSORIO, P. & ORTEGA, E. 2004. Signal regulators in FcR-mediated activation of leukocytes? *Trends in immunology*, 25**,** 529-535.

MISHRA, S. K., MOHANTY, S., PATI, S. S. & SAHU, P. K. 2007. Diagnostic Approach to Malaria. *Clinical Medicine a Practical Manual for Studies & practicals by Agarwal*, 1**,** 409.

MOCKENHAUPT, F. P., EHRHARDT, S., BURKHARDT, J., BOSOMTWE, S. Y., LARYEA, S., ANEMANA, S. D., OTCHWEMAH, R. N., CRAMER, J. P., DIETZ, E. & GELLERT, S. 2004. Manifestation and outcome of severe malaria in

children in northern Ghana. *American Journal of Tropical Medicine and Hygiene,* 71**,** 167-172.

MOLD, C., GEWURZ, H. & DU CLOS, T. W. 1999. Regulation of complement activation by C-reactive protein. *Immunopharmacology,* 42**,** 23-30.

MOSTOV, K. E. & DEITCHER, D. L. 1986. Polymeric immunoglobulin receptor expressed in MDCK cells transcytoses IgA. *Cell,* 46**,** 613-621.

MURPHY, S. C. & BREMAN, J. G. 2001. Gaps in the childhood malaria burden in Africa: cerebral malaria, neurological sequelae, anemia, respiratory distress, hypoglycemia, and complications of pregnancy. *American Journal of Tropical Medicine and Hygiene,* 64**,** 57-67.

MUTABINGWA, T. K., BOLLA, M. C., LI, J. L., DOMINGO, G. J., LI, X., FRIED, M. & DUFFY, P. E. 2005. Maternal malaria and gravidity interact to modify infant susceptibility to malaria. *PLoS medicine,* 2**,** e407.

NATVIG, J. B. & KUNKEL, H. G. 1973. Human immunoglobulins: classes, subclasses, genetic variants, and idiotypes. *Advances in Immunology,* 16**,** 1-59.

NEBIE, I., DIARRA, A., OUEDRAOGO, A., SOULAMA, I., BOUGOUMA, E. C., TIONO, A. B., KONATE, A. T., CHILENGI, R., THEISEN, M., DODOO, D., REMARQUE, E., BOSOMPRAH, S., MILLIGAN, P. & SIRIMA, S. B. 2008. Humoral responses to Plasmodium falciparum blood-stage antigens and association with incidence of clinical malaria in children living in an area of seasonal malaria transmission in Burkina Faso, West Africa. *Infection and Immunity,* 76**,** 759-66.

NEZLIN, R. 1990. Internal movements in immunoglobulin molecules. *Advances in immunology,* 48**,** 1-40.

NIELSEN, L. K., GREEN, T. H., NORDERHAUG, L., SANDLIE, I. & DZIEGIEL, M. H. 2007. Functional in vitro studies of recombinant human immunoglobulin G and immunoglobulin A anti-D. *Transfusion,* 47**,** 306-315.

NIMMERJAHN, F. & RAVETCH, J. 2008 Fcgamma receptors as regulators of immune Responses. . *Nature Reviews Immunology,* 8: **,** 34–47.

O'MEARA, W. P., MANGENI, J. N., STEKETEE, R. & GREENWOOD, B. 2010. Changes in the burden of malaria in sub-Saharan Africa. *The Lancet infectious diseases,* 10**,** 545-555.

OCHOLA, L., VOUNATSOU, P., SMITH, T., MABASO, M. & NEWTON, C. 2006. The reliability of diagnostic techniques in the diagnosis and management of malaria in the absence of a gold standard. *The Lancet infectious diseases,* 6**,** 582-588.

OEUVRAY, C. H., BOUHAROUN-TAYOUN H, GRAS-MASSE E, BOTTIUS T, KAIDOH M, AIKAWA M. C, FILGUEIRA A & TARTAR P. D 1994. Merozoite surface protein-3: a malaria protein inducing antibodies that promote Plasmodium falciparum killing by cooperation with blood monocytes. *Blood,* 84**.,** 1594–1602.

OWUSU-AGYEI, S., ASANTE, K. P., ADJUIK, M., ADJEI, G., AWINI, E., ADAMS, M., NEWTON, S., DOSOO, D., DERY, D. & AGYEMAN-BUDU, A. 2009. Epidemiology of malaria in the forest-savanna transitional zone of Ghana. *Malaria Journal,* 8**,** 220.

OWUSU-AGYEI, S., FRYAUFF, D. J., CHANDRAMOHAN, D., KORAM, K. A., BINKA, F. N., NKRUMAH, F. K., UTZ, G. C. & HOFFMAN, S. L. 2002. Characteristics of severe anemia and its association with malaria in young children

of the Kassena-Nankana District of northern Ghana. *The American journal of tropical medicine and hygiene,* 67**,** 371-377.

OZER, Y. M. 2012. *A Student Guide to Health: Understanding the Facts, Trends, and Challenges,* ABC-CLIO.

PARHAM, P. 2009. *The immune system,* Garland Science.

PLEASS, R. 2009. Fc-receptors and immunity to malaria: from models to vaccines. *Parasite immunology,* 31**,** 529-538.

PUCK, J. M., GLEZEN, W. P., FRANK, A. L. & SIX, H. R. 1980. Protection of infants from infection with influenza A virus by transplacentally acquired antibody. *Journal of Infectious Diseases,* 142**,** 844-849.

QUIE, P. Antimicrobial defenses in the neonate. Seminars in perinatology, 1990. 2-9.

RAGHAVAN, M. & BJORKMAN, P. J. 1996. Fc receptors and their interactions with immunoglobulins. *Annual review of cell and developmental biology,* 12**,** 181-220.

RAI, N. & ABRAHAM, J. 2012. Different Clinical Features of Malaria. *Asian Journal of Biomedical and Pharmaceutical Sciences,* 2**,** 28-31.

RAMSEY, J. M., TELLO, A., CONTRERAS, C. O., ORDOÑEZ, R., CHIRINO, N., ROJO, J. & GARCIA, F. 2002. Plasmodium falciparum and P. vivax Gametocyte-Specific Exoantigens Stimulate Proliferation of TCR γδ+ Lymphocytes. *Journal of Parasitology,* 88**,** 59-68.

RANTALA, A.-M., TAYLOR, S. M., TROTTMAN, P. A., LUNTAMO, M., MBEWE, B., MALETA, K., KULMALA, T., ASHORN, P. & MESHNICK, S. R. 2010. Comparison of real-time PCR and microscopy for malaria parasite detection in Malawian pregnant women. *Malaria Journal,* 9**,** 269.

RAVETCH, J. V. & BOLLAND, S. 2001. IgG Fc receptors. *Annual review of immunology,* 19**,** 275-290.

RAVETCH, J. W. & CLYNES, R. A. 1998. Divergent roles for Fc receptors and complement in vivo. *Annual review of immunology***,** 16:421.

REDPATH, S., MICHAELSEN, T. E., SANDLIE, I. & CLARK, M. R. 1998. The influence of the hinge region length in binding of human IgG to human Fcγ receptors. *Human immunology,* 59**,** 720-727.

REILING, L., RICHARDS, J. S., FOWKES, F. J. I., WILSON, D. W., CHOKEJINDACHAI, W., BARRY, A. E., THAM, W. H., STUBBS, J., LANGER, C. & DONELSON, J. 2012. The Plasmodium falciparum Erythrocyte Invasion Ligand Pfrh4 as a Target of Functional and Protective Human Antibodies against Malaria. *PloS one,* 7**,** e45253.

RILEY, E., BENNETT, S., JEPSON, A., HASSAN-KING, M., WHITTLE, H., OLERUP, O. & CARTER, R. 1994. Human antibody responses to Pfs 230, a sexual stage-specific surface antigen of Plasmodium falciparum: non-responsiveness is a stable phenotype but does not appear to be genetically regulated. *Parasite immunology,* 16**,** 55-62.

RILEY, E., WAGNER, G., AKANMORI, B. & KORAM, K. 2001. Do maternally acquired antibodies protect infants from malaria infection? *Parasite immunology,* 23**,** 51-59.

RILEY, E., WAGNER, G., OFORI, M., WHEELER, J., AKANMORI, B., TETTEH, K., MCGUINNESS, D., BENNETT, S., NKRUMAH, F. & ANDERS, R. 2000. Lack of association between maternal antibody and protection of African infants from malaria infection. *Infection and immunity,* 68**,** 5856-5863.

RILEY, E. M. & STEWART, V. A. 2013. Immune mechanisms in malaria: new insights in vaccine development. *Nature medicine,* 19**,** 168-178.

ROGERS, W. O., ATUGUBA, F., ODURO, A. R., HODGSON, A. & KORAM, K. A. 2006. Clinical case definitions and malaria vaccine efficacy. *Journal of Infectious Diseases,* 193**,** 467-473.

ROUSSILHON, C., OEUVRAY, C., MÜLLER-GRAF, C., TALL, A., ROGIER, C., TRAPE, J. F., THEISEN, M., BALDE, A., PÉRIGNON, J. L. & DRUILHE, P. 2007. Long-term clinical protection from falciparum malaria is strongly associated with IgG3 antibodies to merozoite surface protein 3. *PLoS medicine,* 4**,** e320.

ROUX, K. H., STRELETS, L., BREKKE, O. H., SANDLIE, I. & MICHAELSEN, T. E. 1998. Comparisons of the ability of human IgG3 hinge mutants, IgM, IgE, and IgA2, to form small immune complexes: a role for flexibility and geometry. *The Journal of Immunology,* 161**,** 4083-4090.

ROUX, K. H., STRELETS, L. & MICHAELSEN, T. E. 1997. Flexibility of human IgG subclasses. *Mian Yi Xue Za Zhi* 159**,** 3372–3382.

SCHMID-HEMPEL, P. 2009. Immune defence, parasite evasion strategies and their relevance for 'macroscopic phenomena'such as virulence. *Philosophical Transactions of the Royal Society B: Biological Sciences,* 364**,** 85-98.

SCHOFIELD, L. & GRAU, G. E. 2005. Immunological processes in malaria pathogenesis. *Nature Reviews Immunology,* 5**,** 722-735.

SCHUR, P. H. 1987. IgG subclasses--a review. *Annals of allergy,* 58**,** 89-96, 99.

SIMISTER, N. E., STORY, C. M., CHEN, H. L. & HUNT, J. S. 1996. An IgG-transporting Fc receptor expressed in the syncytiotrophoblast of human placenta. *European journal of immunology,* 26**,** 1527-1531.

SNOUNOU, G. & SINGH, B. 2002. Nested PCR analysis of Plasmodium parasites. *Malaria Methods and Protocols.* Humana Press: Springer.

SNOW, R., NAHLEN, B., PALME, R. A., DONNELLY, C., GUPTA, S. & MARSH, K. 1998. Risk of severe malaria among African infants: direct evidence of clinical protection during early infancy. *Journal of Infectious Diseases* 177**,** 819–822.

SNOW, R. W., GUERRA, C. A., NOOR, A. M., MYINT, H. Y. & HAY, S. I. 2005. The global distribution of clinical episodes of Plasmodium falciparum malaria. *Nature,* 434**,** 214-217.

SNOW, R. W., TRAPE, J.-F. & MARSH, K. 2001. The past, present and future of childhood malaria mortality in Africa. *TRENDS in Parasitology,* 17**,** 593-597.

SOE, S., THEISEN, M., ROUSSILHON, C. & DRUILHE, P. 2004. Association between protection against clinical malaria and antibodies to merozoite surface antigens in an area of hyperendemicity in Myanmar: complementarity between responses to merozoite surface protein 3 and the 220-kilodalton glutamate-rich protein. *Infection and immunity,* 72**,** 247-252.

SOLOMON, A. & WEISS, D. T. 1995. Structural and functional properties of human lambda-light-chain variable-region subgroups. *Clinical and diagnostic laboratory immunology,* 2**,** 387-394.

SOOD, D., KUMAR, S., SINGH, S., SHARMA, S., SOKHEY, J. & SINGH, H. 1995. Transplacental immunity and waning of maternal antibody in measles. *The Indian Journal of Pediatrics,* 62**,** 95-99.

STANISIC, D. I., RICHARDS, J. S., MCCALLUM, F. J., MICHON, P., KING, C. L., SCHOEPFLIN, S., GILSON, P. R., MURPHY, V. J., ANDERS, R. F. & MUELLER, I. 2009. Immunoglobulin G subclass-specific responses against Plasmodium falciparum merozoite antigens are associated with control of parasitemia and protection from symptomatic illness. *Infection and immunity,* 77**,** 1165-1174.

STEKETEE, R. W. & CAMPBELL, C. C. 2010. Impact of national malaria control scale-up programmes in Africa: magnitude and attribution of effects. *Malaria Journal,* 9**,** 299.

SUTHERLAND, C. J., TANOMSING, N., NOLDER, D., OGUIKE, M., JENNISON, C., PUKRITTAYAKAMEE, S., DOLECEK, C., HIEN, T. T., DO ROSÁRIO, V. E. & AREZ, A. P. 2010. Two nonrecombining sympatric forms of the human malaria parasite Plasmodium ovale occur globally. *Journal of Infectious Diseases,* 201**,** 1544-1550.

TA, T. H., HISAM, S., LANZA, M., JIRAM, A. I., ISMAIL, N. & RUBIO, J. M. 2014. First case of a naturally acquired human infection with Plasmodium cynomolgi. *Malaria journal,* 13**,** 68.

TAO, M.-H., CANFIELD, S. M. & MORRISON, S. L. 1991. The differential ability of human IgG1 and IgG4 to activate complement is determined by the COOH-

terminal sequence of the CH2 domain. *The Journal of experimental medicine,* 173**,** 1025-1028.

TAYLOR, T. E., FU, W. J., CARR, R. A., WHITTEN, R. O., MUELLER, J. G., FOSIKO, N. G., LEWALLEN, S., LIOMBA, N. G. & MOLYNEUX, M. E. 2004. Differentiating the pathologies of cerebral malaria by postmortem parasite counts. *Nature medicine,* 10**,** 143-145.

THEISEN, M., SOE, S., BRUNSTEDT, K., FOLLMANN, F., BREDMOSE, L., ISRAELSEN, H., MADSEN, S. M. & DRUILHE, P. 2004. A Plasmodium falciparum GLURP–MSP3 chimeric protein; expression in Lactococcus lactis, immunogenicity and induction of biologically active antibodies. *Vaccine,* 22**,** 1188-1198.

THEISEN, M., SOE, S., OEUVRAY, C., THOMAS, A. W., VUUST, J., DANIELSEN, S., JEPSEN, S. & DRUILHE, P. 1998. The glutamate-rich protein (GLURP) of Plasmodium falciparum is a target for antibody-dependent monocyte-mediated inhibition of parasite growth in vitro. *Infection and Immunity,* 66**,** 11-17.

TOLLE, R., FRÜH, K., DOUMBO, O., KOITA, O., N'DIAYE, M., FISCHER, A., DIETZ, K. & BUJARD, H. 1993. A prospective study of the association between the human humoral immune response to Plasmodium falciparum blood stage antigen gp190 and control of malarial infections. *Infection and immunity,* 61**,** 40-47.

TROYE-BLOMBERG, M., WORKU, S., TANGTEERAWATANA, P., JAMSHAID, R., SÖDERSTRÖM, K., ELGHAZALI, G., MORETTA, L., HAMMARSTRÖM, M. & MINCHEVA-NILSSON, L. 1999. Human gamma delta T cells that inhibit the in vitro growth of the asexual blood stages of the Plasmodium falciparum parasite

express cytolytic and proinflammatory molecules. *Scandinavian journal of immunology,* 50**,** 642-650.

VLADUTIU, A. O. 2000. Immunoglobulin D: properties, measurement, and clinical relevance. *Clinical and diagnostic laboratory immunology,* 7**,** 131-140.

WAAIJENBORG, S., HAHNÉ, S. J., MOLLEMA, L., SMITS, G. P., BERBERS, G. A., VAN DER KLIS, F. R., DE MELKER, H. E. & WALLINGA, J. 2013. Waning of maternal antibodies against measles, mumps, rubella, and varicella in communities with contrasting vaccination coverage. *Journal of Infectious Diseases,* 208**,** 10-16.

WAGNER, B., FLAMINIO, J. B., HILLEGAS, J., LEIBOLD, W., ERB, H. N. & ANTCZAK, D. F. 2006. Occurrence of IgE in foals: evidence for transfer of maternal IgE by the colostrum and late onset of endogenous IgE production in the horse. *Veterinary immunology and immunopathology,* 110**,** 269-278.

WHO 2010. Africa Update World Malaria Day *Africa Update*

WHO 2011a. Global Malaria Programme-Questions and Answers *World Malaria Report.*

WHO 2011b. global malaria report. *FACT SHEET, Embargoed until 14:00 CET.*

WINTER, W. E., HARDT, N. S. & FUHRMAN, S. 2000. Immunoglobulin E: importance in parasitic infections and hypersensitivity responses. *Archives of pathology & laboratory medicine,* 124**,** 1382-1385.

WITNEY, A. A., ANTHONY, R. M. & CARUCCI, D. J. 2002. Quantitation of Liver-Stage Parasites by Automated TaqMan® Real-Time PCR. *Malaria Methods and Protocols.* Springer.

ZABRISKIE, J. B. 2009. *Essential clinical immunology*, Cambridge University Press.

http://www.niaid.nih.gov/topics/immuneSystem, 28 February 2013.

http://www.google. com.gh/imgres, 4 January, 2014.