# IMMUNOLOGICAL AND GENETIC CORRELATES OF IMMUNITY TO *PLASMODIUM FALCIPARUM* MALARIA

**BY**



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**B.Sc. BIOCHEMISTRY (Hons)**

**A THESIS SUBMITTED TO THE DEPARTMENT OF BIOCHEMISTRY AND BIOTECHNOLOGY, KWAME NKRUMAH UNIVERSITY OF SCIENCE AND TECHNOLOGY, KUMASI IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF PHILOSOPHY (M.PHIL.) IN BIOCHEMISTRY**

**NOVEMBER 2010**

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# DECLARATION

I hereby declare that except for references to other people's work, which have duly been acknowledged, this thesis is the result of my own research conducted at the Immunology Department of Noguchi Memorial Institute for Medical Research; Accra-Ghana and at the Department of Clinical Biochemistry & Immunology of Staten Serum Institut (SSI); Copenhagen-Denmark, under the supervision of Dr. Daniel Dodoo (Immunology Department of Noguchi Memorial Institute for Medical Research) and Dr. Fareed Kow N. Arthur (Biochemistry Department, Kwame Nkrumah University of Science and Technology). Neither all nor parts of this thesis have been presented for another degree elsewhere.

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# DEDICATION

This work is dedicated to The Lord God Almighty, the source of all wisdom and inspiration. I also dedicate it to my parents Prince Daniel Adu Kofi and Victoria Oforiwaa for their immense support and encouragement throughout my education.

# ACKNOWLEDGEMENTS

All the praise, glory and honour be to the Almighty God for providing tremendous strength, protection, insight and grace every step throughout my entire study and for seeing me through yet another phase of my education.

I am forever grateful to my supervisors Dr. Daniel Dodoo and Dr. Fareed Kow N. Arthur for their expert pieces of advice, constructive criticisms and invaluable suggestions which have contributed to the successful completion of this work. I thank Dr. Daniel Dodoo for his mentorship and guidance during my stay at the Immunology Department and Dr. Ben Gyan, the head of Immunology Department-NMIMR and Dr. Michael Ofori for their immense support and contribution during my work.

This work would have taken forever to complete with the invaluable contribution of Naana, Nana Yaa, Rebecca, Eva, David, Irene, Eunice my special ELISA team. The Lord will surely reward each one of you greatly for all the sacrifices you made for this work. I also express my utmost appreciation to all the colleagues and staff of the Immunology Department-NMIMR especially, Helena, Dorothy, Eric, Kakra, Gerald, Andrea and Sophia for the many ways they contributed to this work. I am deeply grateful to you all.

Special thanks also go to all the study participants and their parents or guardians of Asutsuare and the Estate, Volivo, Osuwem-Gbese and Lanor, Avakpo, Kewum-Atrobinya and Mafikorpe without who this study wouldn't have been possible. I am also sincerely grateful to field assistant coordinator Magnus, and all the field workers who visited the participants weekly to gather morbidity data; Anthoinette, Felicia, Nicolas, Bless, Magdalene, James, Bernice, Gladys, Gideon, Nelson, Bismark, Isaac, Rosemary and Benedicta God bless you all. I also thank the Medical Assistants of Asutsuare and Osuwem, Madam Mary and Madam Gladys respectively and their nurses for examining and managing all the patients during the study. I am indebted to all the laboratory technicians at the Asutsuare Health Centre, Jones, Josephine, Isaac, Moses and Isaac who were very helpful in the collection of clinical data and samples during morbidity surveys. I am also very grateful to the Transport Officer of NMIMR, Mr. Adams and all the drivers of the Institute especially, Mr. Amoah, Pastor Alex, Bismark, Mr. Opare, Mr. Danso for their support during the Field work.

I also thank Dr. Michael Christiansen the Head of the Clinical Biochemistry & Immunology Department of Staten Serum Institut (SSI), Copenhagen-Denmark and Dr. Michael Theisen, leader of the GLURP-group SSI/CMP for welcoming me in their laboratories. I am very grateful to Dr. Michael Theisen for the financial support he provided during my stay and work in Copenhagen and for his invaluable technical advice in developing the genotyping assays. I thank Paula and Catherine for their ever listening ears and willingness to make remarkable inputs during the

establishment of the genotyping assays. My heartfelt gratitude also goes to Mette H., and all the laboratory technicians of the Department of Infection Immunology, SSI; most especially Karina, Dennis P., Dennis S., Christine and Kirsten for offering to train me in various PCR techniques and being there always when I needed some assistant in the laboratory. I also enjoyed the times I spent working at CMP with Mette P., Susana, Micha, Prajakta and Una all of the GLURP-group. God richly bless you all mightily. I am also greatly indebted to the Administrative Assistant at CMP, Claire Alberte Charasse for her ever willingness to offer help on all issues, just to make my stay and work in Copenhagen a joyous and successful one.

Finally, I am very grateful to my family and all my friends for their warmth and support throughout my studies and last but not the least my very special friend Miss Araba Incoom for all the help and encouragement which have culminated into this accomplishment.

This thesis was supported by AMANET AIA Phase 2 Project Grant-EC101/2008 and EDCTP Senior Fellowship Grant-TA.2007.40200.012; 2008 both to Dr. Daniel Dodoo.

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| | |
|---|---|
| ADCI | Antibody Dependent Cellular Inhibition |
| AIA | Afro-Immuno Assay |
| AMA | Apical Merozoite Antigen 1 |
| AMANET | Africa Malaria Network Trust |
| ANOVA | One-Way Analysis Of Variance |
| *ARMA* | *Atlas du Risque de la Malaria en Afrique* |
| BC | Before Christ |
| bp | Base Pairs |
| BSA | Bovine Serum Albumin |
| CD | Cluster of Differentiation |
| CDC | Centre for Disease Control |
| CM | Cerebral Malaria |
| CRP | Complement Reactive Protein |
| CSA | Chondroitin Sulphate A |
| DCs | Dendritic Cells |
| DALYs | Disability-Adjusted Life Years |
| DDT | Dichlorodiphenyltrichloroethane |
| DNA | Deoxyribonucleic Acid |

| | |
|---|---|
| dNTP | Deoxyribonucleotide Triphosphates |
| EBA | Erythrocyte Binding Antigen |
| EDCTP | European and Developing Countries Clinical Trials Partnership |
| ELISA | Enzyme Linked Immunosorbent Assay |
| FcγR | Fc Gamma Receptor |
| GIA | Growth Inhibition Assay |
| GLURP | Glutamate-Rich Protein |
| G6PD | Glucose 6 Phosphate Dehydogenase |
| GPI | Glycosyl phosphatidyl inositol |
| Hb | Haemoglobin |
| HLA | Human Leukocyte Antigen |
| *Ibid* | *Ibidem* (Latin: meaning the same place) |
| ICAM-1 | Intercellular Adhesive Molecule |
| IFN | Interferon |
| Ig | Immunoglobulin |
| IL | Interleukin |
| iNOS | Inducible Nitric Oxide Synthatse |
| IPT | Intermittent Presumptive Therapy |

| | |
|---|---|
| iRBCs | Infected Red Blood Cells |
| ITAM | Immunoreceptor Tyrosine Activation Motif |
| ITIM | Immunoreceptor Tyrosine Inhibitory Motif |
| ITNs | Insecticides Treated Nets |
| MARA | Mapping Malaria Risk in Africa |
| MBL | Mannose-Binding Lectin |
| MHC | Major Histocompatibility Complex |
| MOH | Ministry of Health |
| MSP | Merozoite Surface Protein |
| NK | Natural Killer |
| NMIMR | Noguchi Memorial Institute for Medical Research |
| OPD | Out Patient Department |
| PAMPs | Parasite Associated Molecular Patterns |
| PBMCs | Peripheral Blood Mononuclear Cells |
| PBS | Phosphate Buffer Saline |
| PCR | Polymerase Chain Reaction |
| *Pf*EMP1 | *Plasmodium falciparum* Erythrocyte membrane protein 1 |
| RBC | Red Blood Cells |
| RBM | Roll Back Malaria |

| | |
|---|---|
| SAP | Signalling lymphocytic Activation molecule-associated Protein |
| SCID | Severe Combined Immunodeficiency |
| SMA | Severe Malarial Anaemia |
| SNP | Single Nucleotide Polymorphism |
| TAE | Tris-Acetate EDTA |
| $T_h$ | T helper |
| TLR | Toll-Like Receptor |
| TNF | Tumour Necrosis Factor |
| UM | Uncomplicated Malaria |
| UNICEF | United Nation International Children and Educational Fund |
| VCAM1 | Vascular Cell-Adhesion Molecule 1 |
| WBC | White Blood Cells |
| WHO | World Health Organization |

## ABSTRACT

Malaria is a major cause of morbidity and mortality in children under five years old and pregnant women in sub-Saharan Africa. Previous studies have shown that immunity to malaria is mediated by host immunological and genetic factors and parasite genetics. In this study which sought to identify immunological and genetics correlates of immunity against *Plasmodium falciparum* malaria, data from two consecutive malaria transmission community-based cohort studies and a hospital-based malaria case-control were analyzed. The Afro-immuno assay (AIA) enzyme linked immuno-sorbent assay (ELISA) protocol was used to measure baseline isotype IgG and IgM and IgG1-4 subclasses levels to eight (8) malaria antigens GLURP-R0, GLURP-R2, MSP1 hybrid, EBA-175, AMA1-FVO, MSP3-FVO, LR146 and AS202.11. FcγIIA-131H/R, FcγIIB-232I/T, FcγIIIA-158V/F and FcγIIIB-NA1/NA2/SH together with IgG3 hinge region length polymorphisms were genotyped using various polymerase chain reaction (PCR) and sequencing techniques. There was a strong correlation between IgG1 and 3 for MSP3-FVO; IgG2 and 3 for GLURP-R2; IgG1 and 2 for AMA1-FVO and IgG1 and 2 for EBA-175 with age ($0.413 \leq r \leq 0.202$, $p < 0.00001$). In an age adjusted univariate analysis to investigate the association between the antibodies and clinical malaria, IgG1 to MSP3 FVO (IRR=0.83 [95% CI, 0.64, 1.06, p= 0.13]), IgG3 to GLURP R0 (IRR= 0.89 [95% CI, 0.77, 1.03, p=0.12], IgG3 to EBA-175 (IRR=0.88 [95% CI, 0.73, 1.05, p= 0.14]) and IgG4 to MSP3 FVO (IRR=0.81 [95% CI, 0.65, 1.02, p= 0.08]) showed some association with reduced risk to clinical malaria. In a final analysis using a model that comprises, age and the immunological variables with trends

suggestive of protection, only IgG4 to MSP3 FVO (IRR=0.85 [95% CI, 0.66, 1.08, p= 0.19]) and IgG3 to GLURP R0 (IRR= 0.92 [95% CI, 0.79, 1.07, p=0.29] showed persistent trends towards association with protection though statistically insignificant. The FcγRIIIB-NA1/NA1 genotype was significantly associated with reduced risk to uncomplicated malaria (p=0.007) and severe malaria (p=0.0002) while the FcγRIIIB-NA2/NA2 genotype was associated with susceptibility in both cases. There was an over-representation of the heterozygous long-medium (LM) (46.0%) genotype among individuals apparently protected against clinical malaria while the homozygous medium-medium (MM) (13.9%) was under-represented. For all the antigens, the MM and the homozygous long-long (LL) genotypes were associated with the highest and the lowest IgG3 levels respectively. Trends towards protection approaching significance observed for IgG3 to GLURP-R0 might be due to the cytophilic nature of IgG3 enabling effective parasite clearance through ADCI while IgG4 to MSP3-FVO might probably be a surrogate marker to some specific cytokine response critical for protection against malaria. The association of the FcγRIIIB-NA1/NA1 genotype with protection against and the FcγRIIIB-NA2/NA2 with susceptibility to severe and uncomplicated malaria in this study is consistent with the observation that NA1 has a higher affinity for both IgG1 and IgG3 than the NA2. The over-representation of LM among individuals with reduced risk to clinical malaria might be explained by the fact that such individuals benefit from both an efficient L-allele and a perhaps not so efficient but high IgG3 titre inducing M-allele. Findings from this study support the development of a multivalent vaccine from GLURP-R0 and MSP3-FVO. FcγRIIIB-NA1/NA1 and IgG3 hinge region

length polymorphisms have been implicated in this study as potential confounders to be adequately corrected for in malaria vaccine trials and pre-clinical studies to enable accurate interpretation of data.

# CHAPTER ONE

## 4.0 General Introduction



## 4.1 Introduction

Malaria is an infectious disease caused by protozoan parasites of the genus *Plasmodium* and remains a major health burden in endemic countries, with no less than 243 million clinical cases worldwide in 2008 and an estimated 863,000 deaths (WHO, 2009). Globally, an estimated ninety percent of malaria deaths occur in sub-Saharan Africa annually, mostly among young children under the age of five years and pregnant women (WHO, 2002). Malaria is widely distributed geographically, being present in both the subtropics and tropics where environmental conditions are favorable for the parasite multiplication in its *Anopheles* female mosquito vector (Breman *et al.,* 2001; WHO, 2005). Even though presently malaria is considered a disease of the poor and underdeveloped countries, its financial toll on Africa is more than $12 billion annually and in spite of this the disease still accounts for about 25% of all deaths in children under the age of five in Africa (Miller *et al.,* 2002).

Human malaria is caused by obligate intra-erythrocytic *Plasmodium* parasites. Of the over 100 species of *Plasmodium* spp, only five infect humans: *P. malariae, P.*

*ovale*, *P. vivax*, *P. knowlesi* and *P. falciparum* (Russell, 1956; Greenwood *et al.*, 1991; Pain *et al.*, 2008). *Plasmodium malariae* and *Plasmodium ovale* are relatively infrequent causes of morbidity, whereas *Plasmodium vivax* is a common cause of severe, acute febrile illness, especially in Asia and South America, but is rarely fatal (Stevenson and Riley 2004). *Plasmodium knowlesi*, which is a natural parasite of the *Macaca fascicularis* (the 'kra' monkey); is now increasingly been recognized as a significant cause of human malaria, particularly in southeast Asia (Cox-Singh *et al.*, 2008).



However, the vast majority of severe malaria cases and deaths are caused by *Plasmodium falciparum*, which is endemic in most of sub-Saharan Africa and in many other regions of the tropical world (Greenwood *et al.*, 1991; Stevenson and Riley 2004). Malaria could be caused by an infection with only one or a combination of these species. The major means of the parasite transmission is by the bite of an infected female *Anopheles* mosquito, but infections can also occur through exposure to infected blood products (transfusion malaria) and by congenital transmission (Nasr *et al.*, 2008). Infection with the parasite may result in asymptomatic, mild or severe malaria (WHO, 2002). Severe pathology, typically anaemia and/or cerebral malaria, metabolic acidosis accompanied by hypoxia, hypoglycemia and lactic acidosis, resulting from the destruction of erythrocytes, bone-marrow suppression and impaired circulation owing to peripheral hypotension and adherence of infected erythrocytes to the vascular endothelium (Stevenson and Riley 2004). Inflammatory mediators have been repeatedly implicated in the

severity of the disease (Kwiatkowski *et al.,* 1997; Hunt and Grau 2003); giving more emphasis to the widely held belief that severe malaria is, at least in part, an immune-mediated disease (Clark *et al.,* 1987). Severe malaria which generally accounts for about 2% of clinical cases is the most fatal form of the disease and may manifest as severe malaria anaemia (SMA) and/or cerebral malaria (Hill *et al.,* 1991) with fatality occurring within 48 hours of hospitalisation especially in children under five years old (Greenwood *et al.,* 1991).



The general patterns of the disease depend markedly on the age and the previous immunological experience of the host (Baird *et al.,* 1998). In areas of high malaria transmission, the greater burden of disease is borne by infants and young children; life-threatening disease in such an area typically consists of metabolic acidosis (which leads to respiratory distress), cerebral malaria (Hill *et al.,* 1991) and severe malaria anaemia (SMA). However, in areas of lower transmission, primary infections might occur in adulthood, in which severe disease more frequently involves additional disturbances, such as renal failure, pulmonary oedema, shock and jaundice (Schofield and Grau 2005). Although the pathogenesis of malaria has been studied extensively, there are still no clear answers as to why a minority of children (about 1-2%) develop complications like cerebral malaria and die (Hill *et al.,* 1991). The reasons for differences in susceptibility to malaria are multi-factorial and the interplay of factors relating to the parasite, the host, and the environment are thought to be key players. One important factor that modulates the outcome of malaria is host immunogenetics (Clark and Schofield 2000). According to Troye-

Blomberg *et al.*, (1999), immune response to malaria may be similar in all individuals but differences in host genetics may predispose certain individuals to the immunopathologies that lead to severe malaria.

Over the years, several curative and preventive drugs have been developed to help reduce the burden of malaria. However, problems of resistance of the *Plasmodium species* parasite to these drugs and the *Anopheles species* vector to insecticides as well as the inability of the most affected countries to mobilize and sustain the resources needed for effective malaria control programs have prompted the urgent need for an effective vaccine against malaria (Doolan *et al.,* 2003). *P. falciparum* has a complex life cycle characterized by morphologically and antigenically distinct stages that are targeted by stage-specific immunity with antibodies playing a pivotal role (Doolan *et al.,* 2003). Naturally-acquired immunity to malaria has been shown to be non-sterile, a process termed 'premunition' (Bouharoun-Tayoun and Druilhe 1992b), where individuals in endemic populations are protected from clinical disease despite the carriage of some level of parasiteamia. Immunity is also age and exposure-dependent as younger people experience greater morbidity and mortality than adults who have had long-term exposure to the parasite (COHEN *et al.,* 1961; McGREGOR *et al.,* 1963). The fundamental role of antibodies in malaria immunity has been demonstrated by experiments involving the passive transfer of immunoglobulins from malaria-immune adults to malaria-naive children (COHEN *et al.,* 1961; McGREGOR *et al.,* 1963; Sabchareon *et al.,* 1991) as treatment for malaria. Also, maternally derived malaria-specific antibodies have been found to

protect neonates and infants from malaria in the first few months of life (McGREGOR *et al.,* 1963; Sabchareon *et al.,* 1991).

Consequently, several immuno-epidemiological studies have reported associations between levels of antibody to various malaria parasite-specific antigens most of which are polymorphic surface proteins of the parasite or parasite-derived surface antigens on infected erythrocytes (Ndungu *et al.,* 2002), and reduced risk of infection (Riley *et al.,* 1992; Hogh *et al.,* 1995; al Yaman *et al.,* 1996; Theisen *et al.,* 1998; Branch *et al.,* 1998; Dodoo *et al.,* 1999; Oeuvray *et al.,* 2000; Dodoo *et al.,* 2000; Conway *et al.,* 2000; Cavanagh *et al.,* 2004; Polley *et al.,* 2004; Perraut *et al.,* 2005; Osier *et al.,* 2008; Dodoo *et al.,* 2008; Courtin *et al.,* 2009). However, in most cases these studies have often provided conflicting data, with responses to the same antigen(s) appearing to be protective in some studies but not in others (Riley *et al.,* 1992; Hogh *et al.,* 1995; al Yaman *et al.,* 1996; Branch *et al.,* 1998; Conway *et al.,* 2000; Cavanagh *et al.,* 2004; Perraut *et al.,* 2005). These discrepancies may be a consequence of differences in study designs, sample size, reagents used in laboratory experimental procedures among other things. As a result, to date, the precise antigenic target(s) of protective immunity to malaria remain(s) elusive. Thus, there is presently no single correlate of immunity to clinical malaria, and those described do not sufficiently account for the overall variation in susceptibility observed in a population (Mackintosh *et al.,* 2004).

Merozoite surface proteins (MSP) including MSP1, MSP2, MSP3 and the apical membrane antigen – 1 (AMA1) have been implicated in the initial attachment and apical orientation of the parasite during the erythrocyte invasion stage and are therefore considered important targets of protective antibodies (Fowkes *et al.,* 2010). Antibodies to the erythrocyte binding antigen region II (Osier *et al.,* 2008) an important invasion ligand for *P. falciparum* and a leading vaccine candidate antigen which has been rather less studied (Cowman and Crabb 2006) have also been considered to be important in malaria immunity (Okenu *et al.,* 2000) though this has not been confirmed by others (Osier *et al.,* 2008). In addition, other invasion ligands present in the apical organelles such as the erythrocyte binding antigens (EBA 140 and EBA 181) as well as the *P. falciparum* reticulocyte-binding homologues have also been considered targets of protective antibodies (Cowman and Crabb 2006). Also, antibodies to the glutamate rich protein (GLURP) of the parasite have been associated with protection and lower density parasiteamia in some field studies and have also been shown to inhibit parasite growth *in vitro* (Theisen *et al.,* 1998; Oeuvray *et al.,* 2000; Dodoo *et al.,* 2000; Courtin *et al.,* 2009) but the mechanism of action of these antibodies *in vivo* remains unclear (Courtin *et al.,* 2009).

The cytophillic antibodies (Bredius *et al.,* 1994) have been shown to be important in antibody mediated immunity in malaria (Dodoo *et al.,* 2000; Roussilhon *et al.,* 2007; Aubouy *et al.,* 2007; Dodoo *et al.,* 2008). It has been found that IgG3 exhibit length polymorphism in the hinge region (Theisen, unpublished data), however, it

has not yet been established whether any of the polymorphs enhance the *P. falciparum* clearance activity of IgG3 or otherwise.

Leukocyte Fc gamma receptors (FcγR) confer potent cellular effector functions to the specificity of IgG. FcγR - induced leukocyte functions, including antibody-dependent cellular cytotoxicity, phagocytosis, superoxide generation, degranulation, cytokine production and regulation of antibody production, which are essential for host defense and immune regulation (Van Sorge *et al.,* 2003). There are three families of FcγR (FcγRI (CD64), -RII (CD32) and -RIII (CD16) in humans that bind IgG. Of these, FcγRIIa (CD32) is a predominantly low-affinity Fc receptor expressed on monocytes and macrophages, and other immune cells, that binds to all IgG subtypes ($IgG_{1–4}$), (Shi *et al.,* 2001), thereby providing an important link between humoral and cellular malarial immunity. The efficacy of IgG-induced FcγR function displays inter-individual heterogeneity due to genetic polymorphisms of four FcγR subclasses, FcγRIIa (CD32a), FcγRIIIa (CD16a), FcγRIIb (Li *et al.*, 2003), and FcγRIIIb (CD16b). FcγR polymorphisms have been associated with infectious and autoimmune disease, or with disease severity (Van Sorge *et al.,* 2003). Binding of the heavy chain immunoglobulin domain to Fc receptors (FcR) on phagocytic cells is important for protective immunity against malaria (Ouma *et al.,* 2006). A functionally relevant single nucleotide polymorphism (SNP) in exon-4 of the FcγRIIa gene (G494A), results in two co-dominantly expressed allotypes, differing at amino acid position 131 (R/H) of the receptor's extracellular ligand binding domain and thereby altering the affinity of the receptor for the IgG

subclasses (Sinha *et al.,* 2008). FcγRIIa-131H is the only human FcγR that binds IgG2 efficiently. Associations of FcγRIIa-131R/R with protection against clinical malaria and FcγRIIa-131H/H with susceptibility have been reported (Shi *et al.,* 2001; Cooke *et al.,* 2003; Braga *et al.,* 2005). However, this has not been confirmed in recent publications on the FcγRIIa-131H allele (Nasr *et al.,* 2007; Sinha *et al.,* 2008).



### 4.2 Hypothesis

Malaria antigen-specific antibodies may work individually, in synergy and in concert with host immuno-genetic factors to reduce the risk of clinical malaria.

### 4.3 General Objectives

To determine the individual and synergistic role(s) of antibodies to multiple malaria antigens that correlate with protection against or susceptibility to clinical malaria in relation to underlying immuno-genetic factors such as IgG3 hinge region polymorphisms, ABO blood group, FcγR polymorphisms as well as age.

### 4.4 Specific Objectives

1. To measure IgG and IgM and $IgG_{(1-4)}$ subclasses antibody levels to the malaria antigens; GLURP R0, GLURP R2, MSP1 hybrid, MSP3 FVO, EBA175 RII, AS202.11, LR146 and AMA1 FVO in Ghanaian children and

adults and assess their individual or synergistic significance in the outcome of *P. falciparum* infection.

2. To determine the FcγR2A-131H/R, FcγR2B-232I/T, and FcγR3A- 176F/V SNPs genotypes together with the FcγR3B-NA1, NA2 and SH allotypes of the study participants and assess their significance in the outcome of *P falciparum* infection.
3. To determine length polymorphisms of the IgG3 hinge region and assess the relevance of IgG3 hinge region length in malaria immunity.
4. To determine the relationship between $IgG_{(1-4)}$ subclasses antibody levels of the malaria antigens; GLURP R0, GLURP R2, MSP1 hybrid, MSP3 FVO, EBA175 RII, AS202.11, LR146 and AMA1 FVO and the genotypes; FcγR2A-131H/R, FcγR2B-232I/T, FcγR3A-176F/V, FcγR3B-NA1, NA2, SH allotypes and IgG3 hinge region polymorphisms.

# CHAPTER TWO

## 5.0 LITERATURE REVIEW

### 5.1 Malaria Disease

Malaria is an infectious parasitic disease, transmitted through the bite of an infected female Anopheles mosquito. In humans there five sporozoans of the genus Plasmodium responsible for the disease: *Plasmodium knowlesi, P. vivax, P. malariae, P. ovale and P. falciparum* (Russell, 1956; Pain *et al.,* 2008). The severity of the disease depends on the species of infecting parasite and the host's level of acquired immunity as well as the host genetic disposition (WHO, 2005). Almost all severe forms and deaths from malaria are caused by *P. falciparum* but rarely; however, *P. vivax* or *P. ovale* may result in serious complications, debilitating relapses, and even death (Svenson *et al.,* 1995). The most common symptoms of malaria are chills, fever, nausea, general body weakness and anaemia. The major complications of severe malaria include cerebral malaria, pulmonary edema, acute renal failure, severe anaemia, and/or bleeding. Acidosis and hypoglycemia are the most common metabolic complications associated with malaria. Any of these complications can develop rapidly and progress to death within hours or days (WHO, 2000). In tropical countries with a high transmission of malaria (holoendemic areas), severe malaria is predominantly a disease of young children (1 month to 5 years of age).

## 5.2 Malaria History

Malaria has been a major public health problem since pre-historic times and in ancient Chinese medical writings. Emperor Huang Ti describes diseases characterized by high fevers with enlarged spleens. Evidence of the existence of malaria was also documented on clay tablets from Mesopotamia and Egyptian papyri from 1570 BC and Hindu texts as far back as the sixth century BC (Cox 2010). Homer, Empedocles and Hippocrates of the early Greek civilizations as far back as the 400 BC all described the characteristic poor health, fevers with associated splenomegaly seen in people living in marshy places. This observation resulted in the erroneous believe that malaria fevers were caused by miasmas rising from swamps, a notion which persisted for centuries and in the sixteenth century the Italians named the disease *mal'aria* meaning spoiled or evil air (Cox, 2010) . With the discovery of bacteria by Antoni van Leeuwenhoek in 1676, and the incrimination of microorganisms as causes of infectious diseases the search for the cause of malaria intensified. By the year 1879, with the advent of the germ theory of infection by Louis Pasteur and Robert Koch the miasma theory was beginning to phase out and the two theories vying for contention were whether the microorganisms responsible were transmitted (1) by air and inhalation or (2) by water and ingestion. In that same year, the Italian Corrado Tommasi-Crudeli and the German, Theodor Albrecht Edwin Klebs, eminent microbiologists of their time claimed they had isolated a bacterium, *Bacillus malariae*, from some marshes in a

malaria prone area, which when isolated in culture and injected into rabbits caused febrile infections accompanied by enlarged spleens reminiscent of malaria (Klebs and Tommasi-Crudeli, 1888). However, this did not seem to have satisfactorily convinced scientists of the time and in 1880, Charles Louis Alphonse Laveran a French army officer working in Algeria at the time discovered malaria parasites for the first time in 148 out of 200 patients' blood samples he examined. Interestingly, Laveran was using only a dry objective lens with a maximum magnification of x400 but observed several different forms of erythrocytic organism including crescents, spherical motionless bodies with pigment, spherical moving bodies with pigment and bodies that extruded flagella-like structures all of which he thought were on the outside of the red cells. Remarkably, he suggested these were different stages of the organism that began with clear spots that grew, acquired pigment and filled the corpuscle which then burst coinciding with the fevers associated with malaria (Laveran, 1881). He noted that quinine removed these stages from the blood and also mentioned the involvement of the brain in some forms of the disease (Laveran, 1893). Laveran called the parasitic protozoan *Oscillaria malariae* (Laveran, 1881; Cox, 2010). Many scientists became interested in malaria study and by the 1890s, through the work of malariologists such as the Italians, Golgi and Marchiafava among others, three species of the protozoan were identified with specific periodicities and other characteristics responsible for benign tertian (48 hour periodicity) (*Haemamoeba vivax*), malignant tertian (48 hour periodicity) (*Laverania malariae*) and quartan (72 hour periodicity) (*Haemamoeba malariae*) malaria now known as *Plasmodium vivax, P. falciparum and P. malariae*

respectively (Golgi, 1886; Golgi, 1889; Laveran, 1893; Marchiafava and Bignami, 1894). In 1918, John Stephens discovered a fourth species which resembled *P. vivax* when working in West Africa, and described it as *P. ovale* (Stephens, 1922).

The major challenge after discovery of the parasites was to explain how the disease spread from one person to another – the mode of transmission. Many scientists of the time including Laveran, Patrick Manson (who had demonstrated in 1877 that filarial worms were transmitted by mosquitoes) (Manson, 1878), the American physician Albert King who was the proponent of what became known as the 'mosquito – malaria doctrine' (King, 1883) all suggested that mosquitoes might somehow be connected to malaria. However, opinions differed on the exact means of transmission; some investigators, including Manson, thought that humans became infected by drinking water contaminated by infected mosquitoes while others thought that the infection was acquired by inhaling dust from dried-up ponds in which infected mosquitoes had died, thus, still dwelling on some variations of 'the water and ingestion' and 'air and inhalation' theories. Manson also toyed with the idea of mechanical transmission where the parasites were passively carried from host to host on the proboscis of a mosquito. The exact mode of transmission remained elusive to them all until August 20$^{th}$ 1897, Ronald Ross an army surgeon and a colleague of Mason successfully demonstrated that avian malaria parasites (*Plasmodium relictum*) were transmitted by the bite of infected mosquitoes and stated that by implication this would be the case for human malarias (Ross, 1923; Bynum and Ovary, 1998). Unfortunately, due to his duties as an army surgeon, he

could not experimentally prove the transmission of human malaria parasite by the anopheline mosquito until 1899 when the Italian scientists; Grassi, Bigmani and others had already gone ahead to demonstrate this conclusively in 1898 (Grassi *et al.*, 1899).

The sexual stages in the blood were discovered by William MacCallum a medical student and his colleague Eugene Opie in the United States while examining the blood of cows infected with a haematozoan, *Haemoproteus columbae*, a closely related parasite to the human malaria parasite in 1897. They observed the fusion of the male and female gametes (formation of ookinete) of *H. columbae* and correctly stated that the human malaria parasite will have the same sexual stages in its life cycle (MacCallum, 1898). In 1948, Henry Shortt and Cyril Garnham discovered that malaria parasites developed in the liver before entering the blood stream (Shortt and Garnham, 1948) and Wojciech Krotoski added the final piece of the puzzle to the parasite's life cycle in 1982 by discovering the presence of dormant stages in the liver (Krotoski *et al.*, 1982).

### 5.3 Global Malaria Burden

Over half a century ago Paul Russell wrote in the preface of his book '*Man's Mastery of Malaria'* (Russell, 1955) –

*"While keeping in mind the realities one can nevertheless be confident that malaria is well on its way towards oblivion. Already as a malariologist, I feel premonitory twinges of lonesomeness, and in my own organisation I am now a sort of 'last*

*survivor'. So perhaps it is fitting that I should take this backward glance at the fascinating pages of malaria history"* (Russell, 1955).

In the 55 years that have passed since Russell made this remarkable observation, malaria has remained an important public health problem in more than 88 countries and territories with about 50% of the world's population at risk according to Hay *et al.,* (2004) estimates for 2010. Although largely eliminated from North America and Europe, it remains the most lethal infectious disease in tropical and subtropical regions of the world, infecting about a third of the world's population. The disease is usually restricted to areas with altitudes less than 1,500m and temperature ranges of 24ºC to 32ºC (Janovy and Roberts 1996) where the anopheline mosquito survives. However, demographic and climatic changes due to global warming have been thought to influence the vector and hence parasite distribution (MARA/ARMA, 1998) courting fears of malaria resurgence in areas such as Central Asia and Eastern Europe where malaria had been eradicated (Breman *et al.,* 2001). Hay *et al.,* (2004) estimated that in 2010, about 3.4 billion people worldwide will be at risk of malaria caused by at least one of the five *Plasmodium* species infecting humans. The public health impact of malaria is most crucial in the tropics, especially in sub-Saharan Africa, where it remains the leading cause of morbidity and mortality especially in children and pregnant women. In sub-Saharan Africa, there are between 400 and 900 million acute febrile episodes of malaria yearly with a resultant 0.7 – 2.7 million deaths in children under five years of age (Breman *et al.,* 2001; WHO, 2002). Among children above five years old, an estimated 25-30% of all deaths are attributed to malaria (Payne *et al.,* 1976; Molineaux 1985;

Greenwood *et al.,* 1987), but less than 20% of these deaths come to the attention of any formal health system. The increasing trend of morbidity and mortality can mostly be attributed to drug resistant parasite strains and insecticides-resistant vectors. Malaria is estimated to cost Africa about US $12 billion annually accounting for about 40% of public health expenditure, 30 – 50% of in-patient admissions and up to 50% out-patient visits in areas of high malaria transmission (Gallup and Sachs 2001; Sachs and Malaney 2002).



The heavy toll of malaria on endemic populations has been as a result of inefficient control of the mosquito vectors and resistance of the mosquito vectors as well as parasites to the major classes of insecticides and anti-malaria drugs respectively and the lack of an effective vaccine against the disease (Nacher 2001). In addition, failure to diagnose malaria in the early stages often leads to mortality which could have been avoided by timely treatment (Fischer and Bialek 2002). Furthermore, poverty and malnutrition have been shown to be factors which worsen the disease burden and thus contribute to increasing morbidity and mortality in malaria endemic regions such as sub-Saharan Africa (Kasis *et al.,* 2001). Malaria has been identified to slow down economic growth by reducing the capacity and efficiency of the labour force, thus decreasing productivity with consequent decrease in national income and is also known to discourage tourism and business investment in endemic areas. In 1993, the World Bank ranked malaria as the leading cause of lost disability-adjusted life years (DALYS) in Africa with an estimated 3 million future life years lost from disability and premature death (World Bank, 1993). In 1995,

malaria endemic countries were estimated to have only one-third of the income levels of non-endemic countries globally (Gallup and Sachs 2001) the difference of which may be largely due to the socio-economic burden of malaria.

### 5.3.1 Malaria in Ghana

Malaria is hyperendemic in Ghana and accounts for about 40 - 60% of all Out Patients Department's (OPD) visits in public health facilities (Ministry of Health (MOH), 2002) with 36 – 40% of the affected being children under age 5 years and an estimated 22% of these children die. Between 1995 and 2001 alone, Ghana recorded 2.2 million reported cases of malaria. The disease accounts for 13.2% of all mortalities in Ghana and ranks fifth as the commonest cause of death in children from ages 0 to 4 years (RBM, 2005). Examinations of infections by blood slides show that *P. falciparum* accounts for about 90% of malaria infections in the country while *P. malariae* and *P. ovale* account for about 9.9 and 0.1% respectively (RBM, 2005). *Plasmodium falciparum* is thus the prominent parasite species carried by a combination of vectors. The principal vectors are the *Anopheles gambiae complex*, which is most widespread and difficult to control, and *Anopheles funestus* (Ahmed 1989; MOH, 1991). About 13.8% of pregnant women in Ghana suffer from malaria and about 9.4% of death in pregnant women are malaria related (Antwi and Marfo 1998). There were an estimated 8.3 million malaria cases in 2006 and 3.2 million in 2008 in Ghana. *P. falciparum* was the main parasite responsible and 26% of the reported cases in 2008 were confirmed by microscopy (WHO, 2009). The number of malaria cases did not reduce significantly between 2001 and 2007, but the numbers of reported inpatient cases and deaths increased. The rise in number cases

was perhaps due to better reporting or a change in the incidence of malaria (*ibid*). The 2008 demographic and health survey reported, 33% of households owned an insecticide treated net (ITN), and only 19% of children under 5 had slept under an ITN the previous night (*ibid*). While 24% of febrile children received an antimalarial drug, only 12% were given artemisinin-based combination therapy (ACT) (*ibid*). Funding for malaria control in Ghana is provided by the Government, the Global fund, the World Bank and the United States President's Malaria initiative. Together, these bodies have increased funding for malaria control from almost nothing in 2005 to about US$ 90 million during 2006–2008, with annual expenditure of US$ 30 million (*ibid*). Malaria transmission is usually continuous throughout the year; however, cases increase during and just after the rainy seasons (Ahmed 1989)

It is estimated that sufferers of malaria lose 9.03 workdays per episode and lose 5 days in caring for the sick (Asante *et al.*, 2004). The value of productive time lost to a household is estimated as $8.92 per episode (*ibid).* Similarly, an episode of malaria is estimated to cost a household $15.79 whilst they spend $1.3 a week on insecticides and mosquito coils in an attempt to prevent malaria (*ibid*). Malaria costs the Ministry of Health (Sinha *et al.*, 2008) an approximated $7.7million per annum (*ibid*).

### 5.3.2 Global Malaria Control

Since the discovery of the mode of *Plasmodium* transmission by the anopheline mosquitoes, various intervention measures against malaria have focused primarily on environmental control of mosquito breeding sites (Covell 1941; Russell 1955; Bradley 1992; Gilles and Lucas 1998; Konradsen *et al.,* 2004). Globally, this approach achieved much success in several places such as the Indonesia (SWELLENGREBEL 1950), Malaysia (Watson 1921) some parts of Zambia (Watson 1953; Utzinger *et al.*, 2002), Egypt (Shousha 1948; Killeen *et al.*, 2002) and Brazil (Soper and Wilson 1943; Killeen *et al.,* 2002) and accounted for the conspicuous contraction of global malaria distribution by 1946 (Hay *et al.,* 2004). However, this approach suffered utter failure in places like Sierra Leone and India (Ross 1923; Russell 1955) due to logistics difficulties and lack of commercial incentives for investment (Bradley 1966; Bruce-Chwatt and de Zulueta 1980; de Zulueta 1998). In the 1940s the residual insecticide properties of dichlorodiphenyltrichloroethane (DDT) was discovered and endorsed by WHO which led to the first time, large scale spraying programmes (Najera 1989) to interrupt transmission of the parasite. Together with the administration of chemoprophylaxis to eradicate malaria, the programme achieved much success in many places but with complete disregard of sub-Saharan Africa (Litsios 1966). This led to a further shrinkage of the global malaria distribution from 1946 to 1965 coinciding with the eradication era in many places (Hay *et al.,* 2004).

Since the late 1960s, further malaria control programmes have been embarked on with the national efforts of endemic countries through widespread systematic

control measures such as spraying with DDT, coating marshes with paraffin (to block Anopheles mosquito larvae spiracles), draining stagnant water, and the widespread use of insecticides treated nets (ITNs) and cheap, effective drugs such as chloroquine and pyrimethamine yielding some impressive results (Smith *et al.*, 1977; Yip 1998; White *et al.,* 1999). Despite initial successes, the eradication programmes have not been so successful in many countries due to several factors. Apart from vector resistance to insecticides and parasite resistance to drugs, a combination of social and political factors have also thwarted the malaria eradication campaigns and malaria still remains a significant public health issue in most countries (Hay *et al.,* 2004). Artemisinin-based combination therapy though more expensive than chloroquine, offered greater hope but issues of side effects and emerging resistant strains of *P. falciparum* have been a major concern (Newton and White 1999; Garner and Graves 2005; Jambou *et al.*, 2005).

In 1998 the Roll Back Malaria (RBM) movement was launched as a mentoring, coordinating, and advocacy vehicle for international malaria control (Nabarro and Tayler 1998; Nabarro 1998; Nabarro and Mendis 2000). Its mandate was to reduce the global malaria burden of risk, morbidity, and mortality by half by 2010. Roll Back Malaria has four main targets: to achieve a 60% coverage of children and pregnant women with insecticide treated nets (ITNs), to have 60% of malaria cases receive effective treatment within 24 hours of the onset of symptoms, for 60% of pregnant women to receive intermittent presumptive therapy (IPT), and for 60% of epidemics to be detected within 2 weeks of onset and then responded to

appropriately within a further 2 weeks (WHO/UNICEF, 2003). Inherent in the strategy used to formulate these targets, was a focus on malaria in the highly endemic areas of sub-Saharan Africa where most of the remaining global burden of malaria is found (Mendis *et al.*, 2001; WHO, 1999). Over a decade on, RBM does not seem to have achieved its targeted objectives mainly due to the heavy financial demands of such a programme and this is why the urgent call for an effective malaria vaccine has been much stronger than ever. Unfortunately, current malaria vaccines in clinical trials have so far not yielded satisfactory results primarily due to lack of a better understanding of the mechanism of protective malaria immunity and its effective induction in humans (Kumar *et al.*, 2002; Richie and Saul 2002; Druilhe *et al.*, 2005; Alonso *et al.*, 2005).

### 5.4 The Malaria Parasite and the Mosquito Vector

The protozoan parasite which causes malaria belongs to the genus *Plasmodium* of the phylum Apicomplexa, class Sporzoasida, order Eucoccidiorida, and suborder Haemosporina. There are nine subgenera of the genus *Plasmodium*; three occur in mammals, four in birds, and two in lizards (Garnham 1966). Rodent *Plasmodium* parasites such as *Plasmodium berghei* and also *P. gallinaceum* which infect chicken have been widely studied and have greatly contributed to the present understanding of malaria genetics, immunity and parasite physiology, (Janovy and Roberts 1996). Over 140 species of *Plasmodium* infect reptiles, birds, rodents, bats and primates. Of these, only five infect man: *P. falciparum, P. malariae, P. vivax, P. ovale,* and *P. knowlesi* and they differ in infection characteristics, morphology, geographical

distribution, and relapse pattern. *Plasmodium falciparum* is the most virulent of all the human species being endemic in the tropical and sub-tropical areas and accounts for most cases of complications associated with malaria (Gupta *et al.,* 1994). Unlike other species, *P. falciparum* merozoites can invade erythrocytes of any age, including reticulocytes and this accounts for the unusually higher levels of parasiteamia associated with its infection than the other species (Berendt *et al.,* 1990). *Plasmodium vivax* is widely distributed in the temperate, tropical and sub-tropical regions where it causes substantial morbidity but fewer severe complications (Nosten *et al.,* 1999). *P. vivax* has a remarkable ability to relapse several years after initial infection (Janovy and Roberts 1996). *Plasmodium malariae* causes the quartan malaria and is also associated with renal complications (Garnham 1966; Hendrickse *et al.,* 1972). This species has been found to also infect wild animals such as chimpanzees at about the same rate as humans (Levine 1973) but are considered unimportant as reservoirs because they do not live along side humans. *Plasmodium ovale* is the species that cause ovale or mild tertian malaria and it is the rarest of the five malaria parasites of humans. This species is confined to the tropics but rare in central and eastern Africa. Until recently, *Plasmodium knowlesi* was not considered a human malaria parasite. However, there is now overwhelming evidence that *P. knowlesi* (a malaria parasite with a 24 hour erythrocytic cycle) malaria is a zoonotic disease involving macaque (*Macca* spp.) and leaf (*Presbytis* spp.) monkeys as reservoir hosts with mosquitoes belonging to the Leucosphyrus group of *Anopheles* mosquitoes as vectors in Malaysia and some

parts of Southeast Asia, causing several hundreds of infections each year (Cox-Singh *et al.,* 2008).

## 5.5 Characteristics of Malaria

### 5.5.1 Parasite life cycle and morphology

Naturally, malaria transmission occurs when an infected female anopheline mosquito carrying the malaria-causing parasite inoculates the human host with sporozoites during a blood meal. It is estimated that no fewer than 100 sporozoites are transmitted per infective bite (Vanderberg 1977; Rosenberg *et al.,* 1990; Ponnudurai *et al.,* 1991; Vanderberg and Frevert 2004). Contrary to the traditional view that sporozoites are inoculated directly into the peripheral circulation, a recent study using an improved imaging technology has shown that they are injected into the skin and may remain there for up to 6 hours before about a third enter the lymphatic while the remaining trickle into the blood stream and travel to the liver (Amino *et al.,* 2006; Yamauchi *et al.,* 2007).

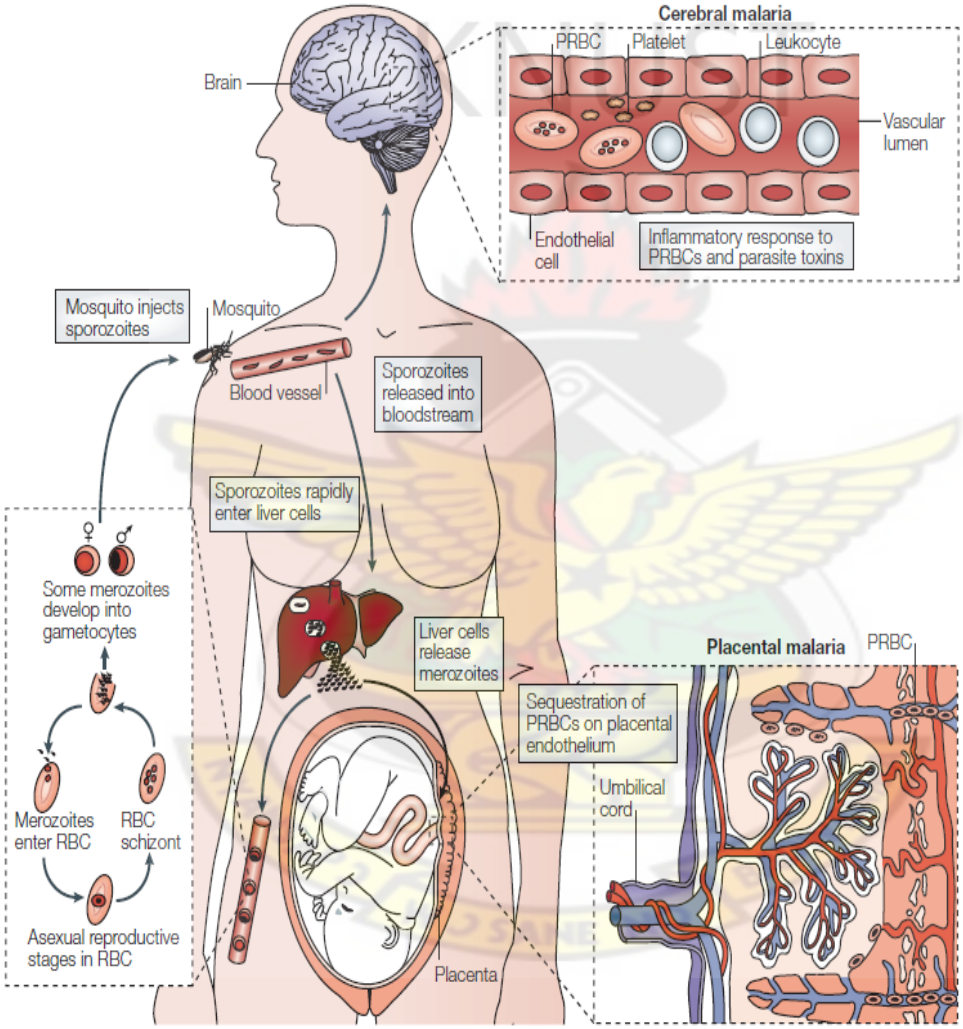



**Figure: 1.0. The life cycle of *Plasmodium falciparum (Schofield and Grau., 2005)***

Some animal studies have shown that not all the inoculated sporozoites leaving the skin have the capacity to successfully result in asexual erythrocytic-stage infection

(Belmonte *et al.,* 2003; Frevert *et al.,* 2005), and in humans it took the bites of at least five *P. falciparum* infected mosquitoes to ensure 100% of volunteers got infected (Rickman *et al.,* 1990; Verhage *et al.,* 2005). It's been now established that injected sporozoites migrate through Kupffer cells and several hepatocytes before finally infecting a hepatocyte (Mota *et al.,* 2002; Ishino *et al.,* 2004) to undergo at least a 2 to 10 days asexual amplification called tissue schizogony, with each schizont containing at least 30,000 uninucleate merozoites (Doolan *et al.,* 2009). In *P. vivax* infection, a dormant form of the parasite called hypnozoites may persist and remain quiescent in the liver for a long time and cause clinical relapse (Krotoski *et al.,* 1982). The liver-stage maturation does not result in clinical symptoms. On schizont rupture, released merozoites with an associated parasitophorous vacuole quickly invade the red blood cells (RBCs) (Cowman and Crabb 2006) to begin the erythrocytic stage (Figure 1.0) of their development which is responsible for clinical symptoms. The immature ring stage parasites (early trophozoites) mature into the late trophozoite stage and then to a schizont undergoing about three to six mitotic divisions to yield 6 to 36 merozoites within each erythrocytic schizont depending on the infecting parasite species (Doolan *et al.,* 2009). In *P. falciparum,* schizonts rupture in 48 hours to release merozoites into the blood stream some of which invade uninfected RBCs and repeat the blood schizogony cycle (*ibid*). Other merozoites differentiate into male and female gametocytes which circulate independently in peripheral circulation about 7 to 15 days after initial invasion of erythrocytes though the stimulating factors of gamecytogenesis remain unclear (Drakeley *et al.,* 2006). In *P. vivax* infection, gametocytes appear prior to the onset

of clinical symptoms while in falciparum malaria they develop within 10 to 40 days after the onset of parasiteamia (Collins and Jeffery 1999; Collins *et al.,* 2004). If an anopheline mosquito ingests these gametocytes during a blood meal, they differentiate into gametes which can combine to form a motile diploid zygote called ookinete within the lumen of the mosquito gut, beginning a process known as sporogony (Doolan *et al.,* 2009). With nourishment from hemolymph, the ookinete differentiates into an oocyst ballooning in size as many thousands of haploid sporozoites are produced through sporogony (*ibid*). The sporozoites are released when the matured oocyst ruptures and they actively migrate to the salivary glands where they rest in saliva bearing channels to be injected into a vertebrate host when the mosquito takes another blood meal (Doolan *et al.,* 2009; Cox 2010).

### 5.5.2 Pre-patent and incubation period

Several factors contribute to the outcome of infection from the time of sporozoite inoculation to either the onset of detectable parasitaemia or malaria symptoms. Anti-malarial prophylaxis and level acquired immunity due to previous exposures can significantly prolong the incubation as well as pre-patent period of infection. In non/semi-immune individuals with *P. falciparum* infection, the pre-disease state where there appear detectable parasitaemia range from 5–10 days, with malaria symptoms occurring between range 6–14 days depending on the effectiveness of both acquired and innate defensive mechanisms as well as residual drug treatments such as anti-malarial prophylaxis (Taylor and Strickland 2000). The incubation

period for both *P. vivax* and *P. ovale* malaria is usually longer and could range from 15–16 days, and may relapse months or years after exposure, due to the presence of hypnozoites in the liver. The longest reported incubation period for *P. vivax* is 30 years (White 2003).

### 5.5.3 Clinical symptoms and infection outcome

Clinical symptoms of malaria are mostly attributable to asexual multiplication of blood stage parasites. The rupture of erythrocytic schizonts during the blood stage infection is associated with a majority of symptoms observed such as fever (>92% of cases), chills (Klebs and Tommasi-Crudeli 1888), headaches (70%), and diaphoresis (Fanger *et al.*, 1997; Genton and D'Acremont 2001). Additional common symptoms include dizziness, malaise, myalgia, abdominal pain, nausea, vomiting, mild diarrhea, and dry cough. Other physical signs may include tachycardia, jaundice, pallor, orthostatic hypotension, hepatomegaly, and splenomegaly (Murphy and Oldfield 1996). The presence of the parasite and the invasion and destruction of RBCs alone might not be sufficient to account for all disease phenotypes seen in malaria. In addition, the release of bioactive parasite molecules upon schizont rupture and an inappropriately regulated host immune response could also be major culprits of fatal pathogenesis, which occurs in only a minority of patients (Schofield and Grau 2005). For instance, it's been shown that fever due to malaria is induced by malaria toxin released upon schizont rupture which induces macrophages to secrete tumor necrosis factor (TNF-α) and interleukin (IL)-1 (Miller *et al.*, 1994). Malaria in endemic regions is often defined

variably as acute febrile illness with or without parasiteamia, depending on the local capacity for parasitological confirmation of diagnosis. Infection is often classified as asymptomatic parasiteamia (parasiteamia with no apparent illness), clinical malaria (febrile episodes) or severe malaria (severe anaemia, cerebral malaria, respiratory distress and hypoglycemia) (White 2003).

## 5.6 Diagnosis



### 5.6.1 Conventional microscopy

Light microscopy of thick and thin stained blood smears remains the 'gold standard' for diagnosing malaria (Moody *et al.,* 2000). Thick smears are 20–40 times more sensitive than thin smears for screening of *Plasmodium* parasites, with a detection limit of 10–50 trophozoites/µl. Thin smears allow one to identify malaria species (including the diagnosis of mixed infections), quantify parasitaemia, and assess for the presence of schizonts, gametocytes, and malarial pigment in neutrophils and monocytes. The diagnostic accuracy relies on the quality of the blood smear and experience of laboratory personnel. The level of parasitaemia may be expressed either as a percentage of parasitized erythrocytes or as the number of parasites per micro-liter of blood. In non-falciparum malaria, parasitaemia rarely exceeds 2%, whereas it can be considerably higher (>5%) in falciparum malaria. In non-immune individuals, hyper-parasitaemia (>5% parasitaemia or >250 000 parasites/µl) is generally associated with severe disease (Torres *et al.,* 2003).

### 5.6.2 Alternative diagnostic methods

Although examination of the thick and thin blood smear is the 'gold standard' for diagnosing malaria, important advances have been made in diagnostic testing, including fluorescence microscopy of parasite nuclei stained with acridine orange, rapid dipstick immunoassay, and polymerase chain reaction assays (Hanscheid and Grobusch 2002). Sensitivity and specificity of some of these methods approach or even exceed those of the thin and thick smear (Killeen *et al.,* 2002). Rapid dipstick immunoassays detect species-specific circulating parasite antigens targeting either the histidine-rich protein-2 of *P. falciparum* or a parasite-specific lactate dehydrogenase. Although the dipstick tests may enhance diagnostic speed, microscopic examination remains mandatory in patients with suspected malaria, because occasionally these dipstick tests are negative in patients with high parasitaemia, and their sensitivity below 100 parasites/µl is low (Moody *et al.*, 2000). Tests based on PCR for species-specific *Plasmodium* genome are more sensitive and specific than other tests, detecting as few as 10 parasites/µl blood (Hanscheid and Grobusch 2002).

### 5.7 Pathogenesis of malaria

Clinical symptoms of malaria are usually as a result of several cascades of events that pertain to the parasite-host interactions. During the erythrocytic stage of the 48-hour life cycle of the parasite, it must adopt means to avoid passage through the spleen as this organ serves as a very efficient filter of infected red blood cells (iRBCs) from circulation (Engwerda *et al.,* 2005). *P. falciparum* erythrocyte membrane protein 1 (*Pf*EMP1) is the name given collectively to members of a

family of variant cell-surface proteins that are encoded by *P. falciparum* and enable iRBCs to engage multiple receptors (Table 1.0) such as intercellular adhesion molecule 1 (ICAM1), vascular cell-adhesion molecule 1 (VCAM1), CD31, CD36, thrombospondin, endothelial-cell selectin (E-selectin), chondroitin sulphate A (CSA) and hyaluronic acid that are expressed by vascular endothelial cells in deep-organ microvascular beds (Schofield and Grau 2005). Binding to these receptors by cell-surface *Pf*EMP1 sequesters parasites and exclude them from the circulation and, consequently, avoiding passage through the spleen. This parasite survival strategy has pathological consequence on the host by concentrating parasites in various target organs depending on the differential expression of the various *Pf*EMP1 members and their diverse endothelial-cell-expressed receptors (Schofield and Grau 2005). The sequestered parasites mature and produce a variety of bioactive molecules that either upregulate or downregulate pathogenic processes, largely through their effects on the innate immune system. Immune responses to infectious agents are mainly initiated by the interaction of pathogen-associated molecular patterns (PAMPs) with receptors expressed by host cells.

**Table 1.0 Malaria products and their bioactivities**

| Parasite product | Receptor and cell type | Pathological and cellular effects |
|---|---|---|
| *Plasmodium falciparum* EMP1-family members | ICAM1, VCAM1, CD36, thrombospondin, E-selectin, chondroitin sulphate A, hyaluronic acid and CD31 on endothelial cells and trophoblast cells; CD36 on DCs | Binding directs parasite to the brain, placenta and possibly other target organs; CD36. Engagement proposed to suppress DC and macrophage activation |
| GPI | TLR2, TLR4 and/or possibly C-type lectins on several cell types, including DCs, macrophages, endothelial cells and adipocytes; CD1d and Vα14–Vβ8 TCR on NKT cells | Induces widespread expression of genes encoding pro-inflammatory proteins (including TNF, IL-1, IL-6, IL-12, iNOS, ICAM1, VCAM1); activates NKT cells; induces TH1- or TH2-cytokine production |
| Haemozoin | TLR9 on DCs | Contradictory reports: both TH1- and TH2- cell activities; induces and inhibits DCs; suppresses macrophages; induces IL-10 production; broadly immunosuppressive |
| Unknown ligands | NKC-encoded receptors on NK and NKT cells | Activates NK cells; induces IFN-γ production; regulates balance of TH1 and TH2 cytokines produced by NKT cells |
| Isopentenyl pyrophosphate | γδ TCRs | Activates γδ T cells; induces IFN-γ production |
| Protein antigens | Diverse TCRs on CD4+ and CD8+ T cells | Activates αβ T cells; induces TH1- or TH2- cytokine production |
| Unknown sugar(s) | MBL in plasma | Possible binding provides protection; low levels of MBL are associated with disease |

(Schofield and Grau 2005)

*P. falciparum* glycosylphosphatidylinositol (GPI) has been shown to function both as a malaria PAMP and as a toxin and it's been found that purified GPI induces the expression of many genes that are implicated in malaria pathogenesis such as the pro-inflammatory cytokines genes encoding tumour-necrosis factor (TNF), interleukin-1 (IL-) and IL-12 (Schofield and Hackett 1993; Tachado *et al.,* 1997; Naik *et al.,* 2000), inducible nitric-oxide synthase (Tachado *et al.,* 1997), and various adhesion molecules that are expressed at the surface of the vascular endothelium and are recognized by *Pf*EMP1, which increases endothelial-cell binding by iRBCs (Schofield *et al.,* 1996).

There are other potential *P. falciparum* PAMPs such as phosphorylated, non-peptidic antigens, to which γδ - T cells respond with slow kinetics (Behr *et al.,* 1996), and haemozoin, the insoluble, crystalline residue of parasite-mediated hemoglobin digestion, which is long-lived and accumulates in phagocytes. Although available reports on haemozoin appear contradictory, it is clear that it has high immunosuppressive properties (Morakote and Justus 1988; Baird *et al.,* 1998). Some studies have implicated haemozoin in either the induction (Coban *et al.,* 2005) or inhibition (Skorokhod *et al.,* 2004) of dendritic cell (Mostov and Deitcher 1986) maturation while others have found it to induce either the production of the T helper 1 ($T_H1$) cytokines TNF (Sherry *et al.,* 1995) and IL-12 (Coban *et al.,* 2005) or the TH2 cytokine IL-10. It also inhibits general proliferative responses by human leukocytes (Deshpande and Shastry 2004) and has been shown to promote monocyte and macrophage dysfunction, by impairing phagocytosis and the

expression of MHC class II molecules, CD11c and ICAM1 (Schwarzer *et al.*, 1992). Induction of fever is among the effects of the overproduction of these cytokines, especially TNF. Tumour necrosis factor (TNF) toxicity can account for most of the symptoms associated with malaria (Clark and Schofield 2000).

### 5.7.1 Severe Malaria



Depending on the nature of transmission dynamics and host age together with host genetics and previous immunological experiences (Baird *et al.*, 1998) malaria could progress from asymptomatic infection to clinical malaria with possible severe complications such as metabolic acidosis, hypoglycemia, anaemia, multiple convulsions and coma resulting in death (Breman *et al.*, 2001). Almost all severe forms and deaths from malaria are caused by *P. falciparum*. It's only rarely, however, that *P. vivax* or *P. ovale* may produce serious complications, debilitating relapses, and even death (Svenson *et al.*, 1995). The World Health Organization (Najera 1989) established criteria for severe malaria in 1990 to assist future clinical and epidemiological studies (WHO, 1990), and in the year 2000, a revision of these criteria was made to include other clinical manifestations and laboratory values that are indicators of a poor prognosis based on clinical experience in semi-immune patients (Table 2.0) (WHO, 2000).

**Table 2.0 Indicators of severe malaria and poor prognosis** (Trampuz *et al.*, 2003)

| Manifestation | Features |
|---|---|
| **Initial World Health Organization criteria from 1990 (WHO, 1990)** | |
| Cerebral malaria | Unrousable coma not attributable to any other cause, with a Glasgow Coma Scale score ≤9. Coma should persist for at least 30 min after a generalized convulsion |
| Severe anaemia | Hematocrit <15% or hemoglobin < 50 g/l in the presence of parasite count >10 000/μl |
| Renal failure | Urine output <400 ml/24 hours in adults (<12 ml/kg/24 hours in children) and a serum creatinine >265 μmol/l (> 3.0 mg/dl) despite adequate volume repletion |
| Pulmonary edema and acute respiratory distress syndrome | The acute lung injury score is calculated on the basis of radiographic densities, severity of hypoxemia, and positive end-expiratory pressure (Gachot *et al.*, 1995) |
| Hypoglycemia | Whole blood glucose concentration <2.2 mmol/l (<40 mg/dl) |
| Circulatory collapse (algid malaria) | Systolic blood pressure <70 mmHg in patients > 5 years of age (< 50 mmHg in children aged 1–5 years), with cold clammy skin or a core-skin temperature difference >10°C |
| Abnormal bleeding and/or disseminated intravascular coagulation | Spontaneous bleeding from gums, nose, gastrointestinal tract, or laboratory evidence of disseminated intravascular coagulation |
| Repeated generalized convulsions | ≥3 convulsions observed within 24 hours |
| Acidemia/acidosis | Arterial pH <7.25 or acidosis (plasma bicarbonate <15 mmol/l) |
| Macroscopic hemoglobinuria | Hemolysis not secondary to glucose-6-phosphate dehydrogenase deficiency |
| **Added World Health Organization criteria from 2000 (WHO, 2000)** | |
| Impaired consciousness<br>Prostration or weakness | Rousable mental condition |
| Hyperparasitemia | >5% parasitized erythrocytes or > 250 000 parasites/μl (in nonimmune individuals) |
| Hyperpyrexia | Core body temperature >40°C |
| Hyperbilirubinemia | Total bilirubin >43 μmol/l (> 2.5 mg/dl) |

Severe malaria which encompasses several complex manifestations is generally categorized into Severe Malaria Aneamia (SMA), Cerebral Malaria (Hill *et al.*, 1991), acute renal failure, and respiratory distress as well as metabolic complications such as acidosis and hypoglycemia (Table 3.0) (Maitland and Marsh 2004). Any of these complications can develop rapidly and progress to death within hours or days (WHO, 2000). In tropical countries with a high transmission of malaria (hyperendemic areas), severe malaria is predominantly a disease of young children (1 month to 5 years of age) (Genton and D'Acremont 2001).

Table 3.0: **Severe and fatal disease syndromes in malaria** (Schofield and Grau 2005).

| Syndrome | Clinical features | Possible sequence or mechanism of disease |
|---|---|---|
| Cerebral malaria | Sustained impaired consciousness, coma, long-term neurological sequelae | Cerebral parasite sequestration; bioactive GPI; pro-inflammatory cytokine cascade; endothelial-cell activation; natural killer T-cell activation; TH1/TH2-cell balance; chemokine production; monocyte, macrophage and neutrophil recruitment; platelet and fibrinogen deposition; CD4+, CD8+ and γδ T-cell involvement; IFN-γ production; neurological metabolic derangements; possibly hypoxia |
| Placental malaria | Placental insufficiency, low birth weight, premature delivery, loss of fetus | *Pf* EMP1-mediated binding to placental endothelium and syncytiotrophoblast through chondroitin sulphate A and hyaluronic acid; cytokine production; chemokine mediated recruitment and infiltration of monocytes; intravascular macrophage differentiation |
| Severe malarial anaemia | Pallor, lethargy, hemoglobin level of 4–6 g per 10 ml | Erythropoietic suppression by toxins and cytokines; increased RBC destruction, owing to parasitization, RBC alterations, complement and immune complex or antigen deposition, erythrophagocytosis, splenic hyperphagism, CD4+ T cells, TH1/$T_H$2 cytokine balance (TNF and IFN-γ versus IL-10) |
| Metabolic acidosis | Respiratory distress, deep breathing (Kussmaul breathing), hypovolaemia | Molecular mechanisms unknown. Possibly widespread parasite sequestration; bioactive toxins; increased vascular permeability; reduced tissue perfusion; anaemia; pulmonary airway obstruction; hypoxia; increased host glycolysis; repressed gluconeogenesis. Some overlap with shock-like syndrome |
| Shock-like syndrome (systemic inflammatory response-like syndrome) | Shock, haemodynamic changes, impaired organ perfusion, disseminated intravascular coagulation | Bioactive toxins; $T_H$1 cytokines; acute-phase reactants |

#### 5.7.1.1 Severe Malaria Anaemia

Severe malaria anaemia (SMA) is the most serious and most common pernicious complication of malaria, and might well be considered the leading cause of deaths from malaria worldwide. Compared with the florid, acute signs of CM, SMA can be chronic or silent. Clinical manifestations of SMA include febrile episodes, hemoglobin concentrations of less than 5g/dl, high levels of parasiteamia often greater than 10,000/ul (Table 2.0) with no evidence of respiratory distress or cardiovascular complications (Maitland and Marsh 2004). The pathogenesis of SMA is not clearly understood as actual mechanisms of pathogenesis may be masked by other factors such as hemoglobinopathies and iron and/or folate deficiencies (Kwiatkowski 1999). However, a marked feature of SMA pathogenesis is the excessive destruction of both iRBCs and non-iRBCs by the spleen and phagocytic cells such as monocytes due deposition of IgG on their surfaces (Abdalla *et al.,* 1980; Adam *et al.,* 1981; Phillips and Warrell 1986; Waitumbi *et al.,* 2000). In some studies, bone marrow suppression (Abdalla *et al.,* 1980; Knuttgen 1987), and inhibition of erythropoiesis (Kurtzhals *et al.,* 1997) have been associated with SMA (Table 3.0). Pro-inflammatory cytokines such as TNF-α which mediate cellular effector functions like phagocytosis have been implicated in the pathogenesis of SMA as they are thought to accelerate the hemolysis of damaged RBCs (Weber *et al.,* 1993). The rapid hemolysis requires that erythropoietic functions of the bone marrow cells are upregulated to compensate for the RBC lost but interestingly, morphological abnormalities of erythropoeitic cells observed in SMA as a result of elevated levels of TNF-α and other pro-inflammatory cytokines

seem to render the cells unresponsive. Consequently, elevated TNF-α level has been found to correlate with high density parasiteamia (Shaffer *et al.,* 1991) while low plasma IL-10/ TNF-α ratio together with reduced IL-10 levels (Kurtzhals *et al.,* 1997; Akanmori *et al.,* 2000; May *et al.,* 2000) have been associated with SMA.

#### 5.7.1.2 Cerebral Malaria



The WHO proposed a definition of cerebral malaria (Hill *et al.,* 1991) as a clinical syndrome characterized by coma (inability to localise a painful stimulus) at least 30 minutes after termination of a seizure or correction of hypoglycaemia, detection of asexual forms of *P. falciparum* malaria parasites on peripheral blood smears, and exclusion of other causes of encephalopathy (WHO, 2000). Cerebral malaria is a major cause of death in children particularly from ages 2 to 4 years living in malaria endemic areas accounting for about 80% of fatal malaria cases (Miller *et al.,* 1994). The pathological processes of *P. falciparum* infection leading to CM is marked by sequestration of iRBCs in cerebral capillaries and post capillary venules (Coltel *et al.,* 2004) which induces flow perturbations that eventually lead to the obstruction and hypoxia of the surrounding brain parenchyma (Turner, 1997). This is the view of the proponents of the mechanical hypothesis in explanation of CM pathogenesis. However, the fact that sequestration of iRBCs in deep microvascular beds occurs routinely in all patients with *P. falciparum* but CM develops in only 1% of these individuals has led to the conclusion that iRBCs sequestration alone might not be sufficient to cause CM but may be necessary (Molyneux 1990; Grau and de

Kossodo 1994; Berendt *et al.,* 1994; Coltel *et al.,* 2004). The opponents of the mechanical hypothesis have argued that the type of unconsciousness due to CM is much similar to sleep, general anaesthesia, and ethanol excess than it has with stroke induced coma (Clark and Schofield 2000). Also, reports show that not all patients with CM have sequestration (Jerusalem *et al.,* 1983). Furthermore, the occurrence of CM at low parasitaemia and the persistence of CM even after the parasite clearance from the blood suggest that other mechanism(s) apart from vascular plugging may be involved (Clark and Schofield 2000). Cerebral malaria studies in humans as well as animal models have revealed that besides iRBCs, other host cells such as leukocytes or platelets (Hunt and Grau 2003; Grau *et al.,* 2003) might also be sequestered in brain microvessels and contribute to pathogenesis either through local effects or distant effects by the production of potentially deleterious mediators such as pro-inflammatory cytokines. Cytokines such as TNF-α, IFN-γ, IL-1, IL-10 and IL-12 secreted by sequestered leukocytes and platelets (Kern *et al.,* 1989; Grau *et al.,* 1990; Schofield and Grau 2005), up-modulate the expression of adhesion molecules such as ICAM-1, VCAM-1, CD36 and E-selectin (Hawrylowicz *et al.,* 1991; Ntoumi *et al.,* 2002) further promoting the pathogenesis.

Proponents of the inflammatory hypothesis postulate that brain vasculature damage is as a result of immune responses directed against sequestering parasites in the brain primarily mediated by TNF and other inflammatory cytokines (Clark *et al.,* 1991). These cytokines lead to the generation of Nitric oxide (NO) which diffuses into the surrounding brain parenchyma where it inhibits glutamate-induced calcium

entry in post-synaptic neurons, thereby suppressing excitatory neurotransmission such that the individual is effectively anaesthetized (*ibid*). This may be a plausible explanation to how malaria could induce deep yet transient coma, but the available clinical evidence does not seem to support this view (Anstey *et al.,* 1996; al Yaman *et al.,* 1996). Tumor necrosis factor has been thought to contribute to the hypoglycemia, which often accompanies cerebral malaria, but this is clearly not the primary cause of the coma in most cases (Kwiatkowski *et al.,* 1990; Brewster *et al.,* 1990; Krishna *et al.,* 1994; Clark *et al.,* 1997). Presently, the general view of CM pathology is largely attributed to a generalized microcirculatory obstruction due to wide spread parasite sequestration in brain microvessels but there are experimental evidence that local and systemic effects of the excessive release of the cytokines that regulate cell-mediated immunity also plays a role (Clark *et al.,* 1989).

### 5.8 Immunity to malaria

Immunity to malaria in humans is very complex mainly because the *Plasmodium* parasite is a complex multistage pathogen and is targeted by multiple immune responses. Besides the complicated life cycle of the parasite which involves different parasitic stages in two different human cell types, hepatocytes and erythrocytes, the *Plasmodium falciparum*'s 23Mb genome also encodes more than 5300 proteins, each of which is a potential target of protective immune responses (Riley *et al.,* 1994; Doolan *et al.,* 2003). Consequently, despite extensive research in malaria immunology, the exact mechanism of immunity to malaria is not fully

understood. What has become clearer, however, is that it includes both specific and non-specific mechanisms, and that both cellular and humoral immune responses are involved (Riley *et al.,* 1994). Malaria naive individuals with no previous experience of malaria almost invariably become ill on their first exposure to the parasite. A febrile illness may develop, which may become severe and, in some cases, may lead to death. Generally, clinical immunity to malaria is a rather slow and gradual process and in malaria-endemic areas, young children are particularly susceptible, with the disease accounting for an estimated quarter of all childhood deaths (Snow *et al.,* 2001). In malaria endemic areas the rate at which natural immunity develops is dependent on the intensity and stability of exposure to *P. falciparum*, with immunity to severe and mild disease developing more rapidly in areas with higher transmission (Gupta *et al.,* 1999; Marsh and Kinyanjui 2006). Epidemiological studies show that, after the initial period in which children are susceptible to severe malaria, development of protective immunity to malaria occurs in three sequential phases: first, immunity to life-threatening disease; second, immunity to symptomatic infection; and only then, third, partial immunity to parasitization (Schofield and Grau 2005). Robert Koch, in 1899, observed that immunity to disease precedes the ability to control parasite densities, an observation subsequently confirmed by others (Christophers 1924; Sinton 1939; McGREGOR *et al.,* 1956) and further strengthened by evidence of anti-toxic immunity emerging from experiments where neurosyphilis patients were treated by deliberate infection with malaria (Molineaux *et al.,* 2002). Thus, strengthening the generally held believe that antitoxic immunity precedes, and is dissociable from, anti-parasite

immunity and that it protects hosts after only a few infections. At the population level, immunity to severe malaria seems to be acquired after only one or two infections (Gupta *et al.*, 1999), although many children with severe disease have a previous history of multiple mild bouts of malaria. Two main approaches have greatly influenced the present knowledge of human immunity to malaria: (1) deliberately inducing malaria in non-immune people, and (2) natural history studies in endemic populations (Fairhurst *et al.*, 2003). Presently, induced malaria in volunteers has become an important aspect of testing of some malaria vaccines and offers the opportunity of detailed studies of possible protective mechanisms (Krause *et al.*, 2007).

Several studies have reported associations between levels of parasite specific antibodies and reduced risk of infection (Polley *et al.*, 2004), but this is not clearly established for disease states such as CM or SMA (Schofield and Grau 2005). So, there is presently no single correlate of clinical immunity to malaria, and those described do not sufficiently account for the overall variation in susceptibility in a population (Mackintosh *et al.*, 2004). However, some studies have shown that antibodies specific for the parasite glycolipid glycosylphosphatidylinositol (GPI) have been found to be negatively associated with a risk of developing SMA (Naik *et al.*, 2000) or CM (Perraut *et al.*, 2005) and with acute febrile episodes (Keenihan *et al.*, 2003), although a cross-sectional study found no association with tolerance for parasitaemia (Boutlis *et al.*, 2002). Although clinical immunity might result from adaptive immune responses to GPI, other explanations include the acquisition of

physiological non-responsiveness to malaria toxins, which is analogous to tachyphylaxis, the process of downregulation of lipopolysaccharide responsive signaling pathways following exposure to the agonist (Schofield and Grau 2005). A further possibility is that disease susceptibility or resistance is regulated to a considerable extent by the $T_H1/T_H2$-cytokine profile of the NK- and NKT-cell component of the immune system (the intermediate between the innate and adaptive immune systems) (Schofield *et al.,* 1999; Hansen *et al.,* 2003a; 2003b; Hansen *et al.,* 2005) or of conventional CD4+ T cells and that clinical immunity is associated with a switch away from the default, $T_H1$-cell-biased responses to $T_H2$-cell-biased responses, which prevents severe disease but controls parasite densities only after an appropriately diverse antibody repertoire is generated (Schofield and Grau 2005). Establishing whether clinical immunity results from adaptive immune responses to bioactive parasite products, physiological desensitization to malaria toxins, regulation of the balance of $T_H1$ and $T_H2$ cytokines, or a combination of mechanisms is critical in malaria vaccine development.

Furthermore, some studies have found merozoite antigens to be important targets of protective antibodies and are thought to function *in vivo* by inhibiting merozoite invasion of erythrocytes, opsonizing merozoites for phagocytosis, and antibody-dependent cellular inhibition (COHEN *et al.,* 1969; Brown *et al.,* 1982; Bouharoun-Tayoun *et al.,* 1990; McCallum *et al.,* 2008). However, it is unclear which merozoite antigens are important targets of naturally acquired immunity. A number of merozoite antigens such as the merozoite surface proteins (MSPs, e.g., MSP1,

MSP2, MSP3, etc) and the apical membrane antigen (AMA1) as well as the glutamate rich protein (GLURP) have established roles in erythrocyte invasion and some have been identified as targets of human invasion-inhibition antibodies or antibody-dependent cellular inhibition *in vitro* (Clark *et al.,* 1989; Theisen *et al.,* 1998; Hodder *et al.,* 2001; O'Donnell *et al.,* 2001; Dutta *et al.,* 2003; Persson *et al.,* 2008). In *P. falciparum* malaria, invasion ligands present in apical membranes, which are considered important in the parasite invasion process, have been shown to be targets of invasion-inhibition antibodies (Cowman and Crabb 2006). These include the erythrocyte binding antigens (e.g., EBA 175, EBA 181 and EBA 140) and the *P.f* reticulocyte-binding homologues. Many of these antigens are currently under evaluation for development into vaccines (Richards and Beeson 2009).

### 5.8.1 Pre-erythrocytic stage immunity

Pre-erythrocytic stage immunity has been thought to be directed against the sporozoites in the circulation and mediated by antibodies, which neutralizes the sporozoite infectivity for hepatocytes (Troye-Blomberg *et al.,* 1999; Pandey 2000). It is a common view that antibody mediated protective immunity is unlikely to be completely effective against pre-erythrocytic stage parasites given the short time the sporozoites spend in circulation before invading hepatocytes (Jefferis and Kumararatne 1990). Although both CD8+ and CD4+ T-cells recognize parasite-derived peptides presented by MHC class I or class II molecules, respectively, on the surface of infected hepatocytes, protection against the pre-erythrocytic stage is mediated primarily by CD8+ T cells (Mostov and Deitcher 1986) together with

cytokines and other factors, such as nitric oxide. It has been shown that i*n vitro* treatment of *Plasmodium* sp.; infected hepatocytes with IFN-γ eliminate *P. falciparum* or *P. berghei* parasites from the cultures (Stokes *et al.,* 1975; Macpherson and Slack 2007). In mice studies where immunization was done with either irradiation attenuated sporozoites or plasmid DNA (Wagner *et al.,* 2006) it has been observed that IL-12 and NK cells are necessary in mounting an effective vaccine–induced immune response. In recent years, an interest in effector CD4+ T cells in anti-malarial immunity has increased and adoptive transfer experiments have shown that CD4+ T cells of the Th1 phenotype could protect against *Plasmodium* sp. challenge *in vivo,* in the absence of any detectable cytotoxicity (Ng *et al.,* 2008). Also, active immunization experiments with linear synthetic peptides, derived from *P. yoelii* proteins, has been found to confer effective protective immunity, which is mediated by CD4+ T-cells and obligatorily dependent on IFN- γ (Grubb 1995; Farouk *et al.,* 2005).

### 5.8.2 Erythrocytic stage immunity

In malaria endemic regions, non–sterile, species-specific , stage - , strain – and variant – (Andrysiak *et al.,* 1986; Fandeur and Chalvet 1998; Rotman *et al.,* 1999) naturally acquired immunity to malaria requires many years of repeated exposure to develop (Baird 1998) and has been shown to be both antibody and cell–mediated.

### 5.8.2.1 Antibody Mediated Immunity

The present insight into erythrocytic stage immunity to malaria was greatly inspired by animal experimentation where B cells and antibodies were found to be crucial, evidenced by the development of chronic *P. chabaudi* parasitaemia in mice lacking B cells as a result of their inability to clear parasites (Linke *et al.,* 1996; von der *et al.,* 1996). In humans, several immune mechanisms have been identified through *in vitro* assays such as the antibody dependent cellular inhibition (ADCI) and growth inhibition assay (GIA) as potentially parasite-neutralizing, and several antigens implicated as targets of protective antibodies, but their relative importance during natural infections *in vivo* is still largely unknown.

Passive transfer of parasite specific monoclonal antibodies (mAb) was shown to confer protection against *P. yoelii* malaria in naive mice (Spencer *et al.,* 1998; Narum *et al.,* 2000). In humans, IgG purified from malaria-immune African adults have been used for treatment of *P. falciparum*-infected non-immune Thai patients and found to demonstrate a reproducible reduction in parasite load and clinical symptoms (Bouharoun-Tayoun *et al.,* 1990). Antibodies against merozoite surface-associated proteins have been thought to function through mechanisms such as blocking the invasion of RBCs (Wahlin *et al.,* 1984) or blocking of merozoite release from schizonts, either by binding to surface-accessible antigens leading to agglutination and inhibition of merozoite dispersal (Lyon *et al.,* 1997) or by entering iRBC through leaky membrane at the time of rupture (Green *et al.,* 1981). In addition, antibodies can also block sequestration of iRBC in internal organs,

presumably allowing for parasite clearance by the spleen (David *et al.,* 1983; Carlson *et al.,* 1990). Further, antibodies to *Pf*EMP-1 (Smith *et al.,* 2000) and the Duffy binding protein (Michon *et al.,* 2000) prevent cytoadherence and may thus prevent rosetting of RBC to iRBC, a crucial process in the pathogenesis of cerebral malaria and thereby help protect against CM (Carlson *et al.,* 1990). The extent of protective immunity found in humans (Chizzolini *et al.,* 1988; Astagneau *et al.,* 1995; Piper *et al.,* 1999), monkeys (Egan *et al.,* 2000) and mice (Hirunpetcharat *et al.,* 1997; 1998) have been shown to correlate with the level of antibodies against asexual blood stage antigens, and are antibody isotype dependent. The IgG subclass responses against ring-infected erythrocyte surface antigen (RESA), merozoite surface protein (MSP) 1, MSP-2 and crude *P. falciparum* antigen in people living in malaria endemic areas are partly determined by host genetic factors and age (Aucan *et al.,* 2001). Cytophilic antibodies of the IgG1 and IgG3 subclasses are considered to be the most important antibodies in protection against *P. falciparum* malaria in humans (*ibid*) and in collaboration with other effector immune cells they may function through antibody-dependent cellular inhibition (ADCI) (Bouharoun-Tayoun and Druilhe 1992a; Bouharoun-Tayoun *et al.,* 1995). The parasite-neutralizing effect obtained in the ADCI assay appeared to mainly be mediated by soluble factors released by the monocytes upon their uptake of opsonized merozoites (Bouharoun-Tayoun *et al.,* 1995). These factors, possibly TNF-α and nitric oxide may function by blocking the division of surrounding intra-erythrocytic parasites. A study also found that monocytes and macrophages may also kill parasites by phagocytosis of opsonized infected erythrocytes (Celada *et al.,* 1983).

In Senegal, a study using crude parasite extracts, found high-avidity cytophilic IgG1 antibodies and IgG3 were predominant in immune subjects (Ferreira *et al.,* 1996) as have been shown by other studies (Bouharoun-Tayoun and Druilhe 1992b; Aribot *et al.,* 1996). Malaria–specific IgG2 levels were found to increase with the age of the subjects in Burkina-Faso, in parallel with the development of protection against infection and malaria disease (Aucan *et al.,* 2000). These antibodies might mediate phagocytosis or lysis of iRBCs by monocytes. The suggested mechanism involves capturing of merozoite or schizont-bound antibodies by Fc receptors on the surface of monocytes (Gysin *et al.,* 1982; Bouharoun-Tayoun *et al.,* 1990). In general, cytophilic antibodies may allow opsonisation and clearance of merozoites or iRBCs by monocytes/macrophages and neutrophils (Taylor *et al.,* 1998; Ndungu *et al.,* 2002).

In acute natural infection, individuals have been shown to often develop highly specific antibody responses to antigens of the infecting parasite variant (Bull *et al.,* 1999; Ahlborg *et al.,* 2002; Franks *et al.,* 2003; Chattopadhyay *et al.,* 2003). As a result, clinical immunity to blood-stage malaria is correlated with the acquisition of a repertoire of antibodies to different variants of parasite antigens, and is related to exposure and age of the host (Dodoo *et al.,* 1999; Bull *et al.,* 2000). Antibodies to merozoite-surface antigens (especially MSP-1, MSP-2 and MSP-3), the apical complex organelles of merozoites [EBA-175, rhoptry protein (Rhop)-1–3, rhoptry-associated protein (RAP)-1–3 and AMA-1], the glutamate rich protein (GLURP) and the dense granule antigen *Pf*155/RESA, all show the capacity to inhibit

merozoite invasion (Berzins and Anders 1999). Furthermore, two other antigens, serine repeat antigen (SERA) and acidic basic repeated antigen (ABRA), which are associated with the merozoite surface at the time of schizont rupture, are found in the immune clusters formed by antibodies inhibiting merozoite dispersal in parasite cultures (Lyon *et al.,* 1989). In addition, antibodies to SERA have been shown to inhibit *P. falciparum* growth *in vitro* and antibodies to ABRA are efficient inhibitors of merozoite invasion (Sharma *et al.,* 1998). Of the parasite-derived antigens expressed on the surface of infected erythrocytes, only Pf332 has been demonstrated to induce parasite-neutralizing antibodies, inhibiting the intra-erythrocytic growth of the parasite (Ahlborg *et al.,* 1996).

However, despite the importance of antibody responses in protection against malaria, there is evidence to suggest that not all antibodies are protective. Polyclonal antibodies specific to MSP2, but not mAb specific to the same antigen, enhance invasion of multiple merozoites into RBC (Ramasamy *et al.,* 1999; 2001). It has also been reported that mAb against $MSP1_{19}$ which inhibit RBC invasion by merozoites and prevent MSP-1 secondary processing, can be blocked by other mAb to the same antigen (Patino *et al.,* 1997). These studies underscore the importance of identifying epitopes that induce protective antibodies when designing a vaccine against malaria.

### 5.8.2.2 Cell-mediated immunity

On the basis of their pattern of cytokine production, CD4 T cells are classified into two major subsets. The T-helper 1 (Th1) subset of cells produce mainly interleukin (IL)-2, interferon (IFN) γ, and tumour necrosis factor (TNF), while on the other hand the T-helper 2 (Th2) cells produce IL-4, IL-5, IL-6 and IL-10 (Abbas *et al.,* 1996) among others. To a greater extent, the Th1 cells are the main subset responsible for cell-mediated immunity (Hill *et al.,* 1991) through the activation of macrophages to release of inflammatory cytokines. The Th2 cells regulate humoral immunity through the facilitation of antibody production and IgG subclass differentiation by B cells (Abbas *et al.,* 1996). T helper 1 cells promote opsonization and phagocytosis by enhancing the production of cytophillic antibodies such as IgG2a and IgG3 in mice, and IgG1 and IgG3 in humans (*ibid*). However, these cells cross-regulate the differentiation and activities of each other through the cytokines they produce. For instance, INF-γ produced by Th1 cells inhibits the development and proliferation of Th2 cells, whereas IL-4 and IL-10 produced by Th2 cells also inhibit the development of Th1 cells. A balance in the activities of these two subsets of T-cells is crucial to protective immunity against blood stage malaria (*ibid*).

Upon encountering malaria antigens in the context of major histocompatibility complex (MHC) molecules on antigen-presenting cells such as dendritic cells (Bruna-Romero and Rodriguez 2001) or macrophages (Chemtai *et al.,* 1984), malaria-specific CD4 T cells bearing αβ T cell receptors (Sayles and Rakhmilevich 1996) are almost readily activated. In mice with severe combined immunodeficiency (SCID), passive transfer of T cells from immune donors was found to suppress *P.*

*chabaudi adami*, infection suggesting that T cells contribute to immunity (van der Heyde *et al.,* 1994). Interestingly, it has been shown that B cell-deficient mice are able to suppress acute infections with *P. c. adami, P. vinkei petteri*, and *P. c. chabaudi* CB at the same rate as normal mice (*ibid*) and this ability was lost when CD4 T cells are depleted using anti-CD4 mAb and the mice succumb to the infection. On the other hand, several studies in *P. c. chabaudi* AS have shown that protective immunity to blood stage malaria requires both CD4 T cells and antibodies and sequential activation of Th1 and Th2 cells is critical for protection (Taylor-Robinson and Phillips 1994; Langhorne *et al.,* 1998).

A study has shown that IFN-γ is the predominant cytokine produced in early acute *P. chabaudi* infection and levels decline with decreasing parasitaemia and is replaced by IL-4 and IL-10 during the latter stages of infection (Taylor-Robinson and Phillips 1994). This suggests that Th1 cells are responsible for control of primary parasitaemia, whereas Th2 cells are required for final clearance of parasites. Therefore, it appears that both Th1 and Th2 are crucial for resistance to malaria contribute to protective immunity at different times of infection, and the balance between these two subsets could be important in determining the outcome of the infection. In humans, the role of CD4 T cells in immunity to malaria is still not well understood. It has been found that both individuals lacking previous exposure to *P. falciparum*, and those already exposed to malaria, have T cells that proliferate and secrete IFNγ in response to parasite antigen and inhibit parasite growth *in vitro* (Fell *et al.,* 1994). Proliferation of peripheral blood mononuclear cells (Deshpande and

Shastry 2004) isolated from malaria-exposed individuals and stimulated *in vitro* with MSP1 or circumsporozoite protein (CSP) was found to correlate with the number of previous malaria episodes (Carvalho *et al.,* 1999). Similarly, levels of *P. falciparum* antigen-specific proliferative responses in children living in highly endemic areas correlated with protection against the parasite (Migot-Nabias *et al.,* 1999).



However, in spite of the critical role of CD 4 T–cells in protection against malaria, there are enough empirical evidence to implicate some aspects of their activity in the pathogenesis of lethal complications. Immuno-deficient mice who received Th1-like T cells were markedly able to suppress parasitaemia following a challenge infection with a non-lethal strain of *P. yoelii*, but this was associated with an increased mortality in recipient mice (Amante and Good 1997). The importance of TNFα in malaria pathogenesis was demonstrated in a study where parasite-infected mice were treated with anti-TNFα antibodies which resulted in prolonged survival, although the mice developed higher parasitaemia. Also depletion of CD4 T cells in mice before infection with *P. berghei* in experimental cerebral malaria was found to prevent convulsions (Hermsen *et al.,* 1998). Hence, CD4 T cells may protect the host by controlling parasitaemia but they can also cause detrimental immunopathology and further studies are necessary to satisfactorily explain the role of these cells in malaria immunity.

The role of T cells bearing γδ TCR in immunity to blood stage malaria remains unclear. A study (Sayles and Rakhmilevich 1996) showed that depletion of γδ T cells does not alter parasitaemia, anaemia or survival rates of mice infected with avirulent *P. c. adami* or virulent *P. c. chabaudi* CB, suggesting that γδ T cells do not contribute to protection (*ibid*). In contrast, others have shown that mice lacking γδ T cells develop chronic parasitaemia following *P. c. chabaudi* AS infection (Langhorne *et al.,* 1995; Seixas and Langhorne 1999). Human γδ T cells isolated from the peripheral blood of malaria non-immune individuals can inhibit growth of the late or extracellular stages of *P. falciparum in vitro* (Troye-Blomberg *et al.,* 1999). Malaria antigen-activated γδ T cells produce mainly, but not exclusively, pro-inflammatory cytokines suggesting that these cells may play a role in both regulatory and cytotoxic functions in malaria pathogenesis (*ibid*). Thus, a role for γδ T cells in providing protective immunity against malaria cannot be excluded; however, further investigations are required.

### 5.9 Human Genetics and Malaria

The genetic basis of resistance or susceptibility to malaria is complex at several levels and it is likely that many different genes are involved and that they interact with environmental variables and with parasite genetic factors. It therefore comes with little surprise that perhaps the greatest number of genes conferring differential susceptibility to any disease has been reported for the various manifestations of malaria (Kwiatkowski 2005).

### 5.9.1 Fc Receptors

The cell surface receptors for the Fc domain of immunoglobulin (Ig) known as Fc receptors (FcRs) are widely expressed on most cells of the hematopoietic system (Ravetch 2003). They play an important role in immune regulation, by serving as the link between antibody-mediated immune responses and cellular effector functions. Specific FcRs have been identified for all Ig classes, including IgA (FcαR), IgD (FcδR), IgE (FcεR), IgG (FcγR), and IgM (FcμR).

Since this work is mainly based on isotype IgG and the subclasses ($IgG_{1-4}$), only FcγR is considered in detail.

#### 5.9.1.1 The Fc Gamma Receptor (FcγR) Family

#### 5.9.1.2 Structure

The classical human FcγRs are encoded by eight genes (Van Der Pol and Van De Winkel 1998) which form part of a clustered gene family located on the long arm of chromosome 1q21–24 (Qiu *et al.,* 1990). There are three families of FcγRs designated, FcγRI (Fanger *et al.,* 1997), FcγRII (CD32) and FcγRIII (CD16), with each containing multiple distinct genes and alternative splicing variants (Kimberly *et al.,* 1995) encompassing at least 12 isoforms. FcγR classes consist of structurally and biochemically distinct molecules which differ not only in cell distribution but also in affinity for IgG subclasses (Van Sorge *et al.,* 2003). The binding of the Fc portion of IgG to FcγRs serves as a crucial link between humoral and cell-mediated immune responses. Recently, it's also been shown that FcγRs also function as

receptors for innate immune opsonins [complement reactive protein (CRP) and signalling lymphocytic activation molecule-associated protein (SAP)] and provide a link between innate and acquired immunity. FcγRs may function as activating receptors (FcγRI, FcγRIIA/C and FcγRIII) or as an inhibitory receptor (FcγRIIB) as they elicit or inhibit immune functions such as phagocytosis, cytotoxicity, degranulation, antigen presentation and cytokine production (Li *et al.,* 2009).



**Table 4.0 Genetic characteristics of human leukocyte FcγR**

| | FcγRI | FcγRII | FcγRIII |
|---|---|---|---|
| Genes | FcγR1A, -B and –C | FcγR2A, -B and -C | FcγR3A and –B |
| Chromosome | 1q21 | 1q23–24 | 1q23–24 |
| Isoforms | IA; IB1; IB2; IC | IIA1; IIA2; IIB1; IIB2; IIB3; IIC | IIIA; IIIB |
| Molecular weight | 72 | 40 | 50–80 |
| Affinity for IgG | High($10^{-8}$-$10^{-9}$/M) | Low (<$10^{-7}$/M) | IIIA: Intermediate ($3x10^{-7}$/M), IIIB: Low (<$10^{-7}$/M) |

(Van Sorge *et al.*, 2003)

The activating FcγRs bear the signaling motif; immunoreceptor tyrosine activation motif (ITAM) while the inhibitory receptor has an immunoreceptor tyrosine inhibitory motif (ITIM) either as associated homodimer accessory chains or on the same ligand binding α-chain (Fig. 2) (Li *et al.*, 2009). In most FcγRs the ligand binding α-chain consists of two or three Ig-like domains in the extracellular region, a transmembrane domain and an intracellular cytoplasmic domain through immune tyrosine activating or inhibitory motifs.

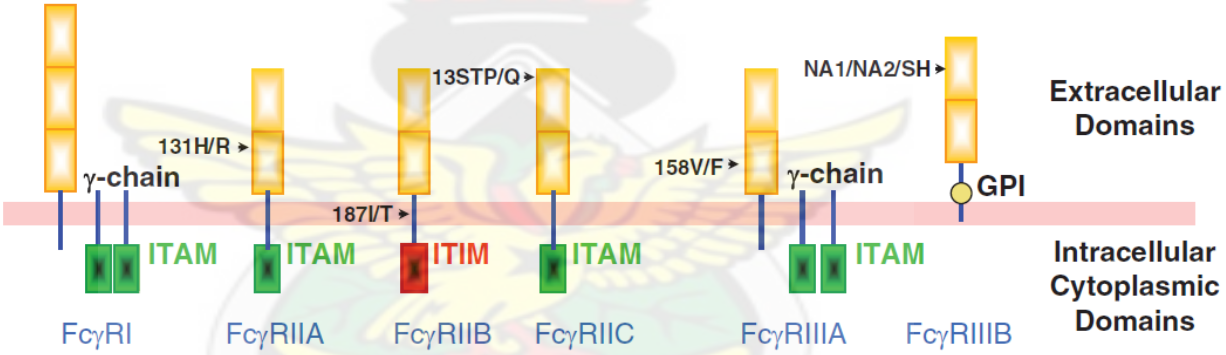



**Figure 2.0** *Structure of human classical Fcγ receptors and location of functionally characterized single-nucleotide polymorphism (SNP) variants (Adapted from Li et al., 2009).*

#### 5.9.1.2.1 FcγRI (CD64)

The *FCGR1* is located in chromosome 1q21 and includes the genes *FCGR1A*, *FCGR1B* and *FCGR1C* (Table 4.0), of which only the FcγR1A product of *FCGRIA* has been identified as a full-length cell surface receptor (Ernst *et al.*, 1992; Hoffmeyer *et al.*, 1997). The functional receptor consists of a single trans-

membrane polypeptide of about 40 KD. FcγRI is the only high- affinity receptor for the Fc domain of monomeric IgG molecules, and binds human IgG1 or IgG3 with a Kd of $10^{-8}$ to $10^{-9}$ M (Ravetch and Kinet 1991). The affinity for IgG2 or IgG4 antibodies is much lower (Table 5.0). The high binding affinity of FcγRI has been attributed to its three extracellular Ig-like domains compared to the others which have only two (Figure 2). The receptor has been found on the surface of monocytes, dendritic cells (DCs), macrophages and also activated neutrophils (Hoffmeyer *et al.*, 1997).



#### 5.9.1.2.2 FcγRII (CD32)

The FcγR2 (CD32) gene subfamily which include *FCGR2A*, *FCGR2B*, and *FCGR2C* are at least three separate genes in humans located in chromosome 1q23 (Table 4.0) (Li *et al.,* 2009). It consists of two extracellular Ig-like domains, which gives it a low binding affinity for monomeric IgG, however, it binds IgG aggregates and immune complexes (ICs) readily. The affinity of FcγRII for human IgG1 or IgG3 is relatively weak with Kd of greater than $10^{-7}$ M, and is essentially nonexistent for IgG2 and IgG4. It is the most widely distributed FcγR and is expressed on monocytes, certain dendritic cells (DCs), neutrophils, B-cells, platelets and natural killer (NK) cells (Capel *et al.,* 1994).

In humans at least nine isoforms of FcγRII may be produced, with the major differences between the isoforms being in the cytoplasmic sequences, although there

are also differences in the extracellular regions, so that some antibodies distinguish FcγRIIA from FcγRIIB isoforms. The FcγRIIB isoforms are the only FcγRs in humans capable of inhibitory signaling and bears an ITIM intracellular region (Daeron *et al.,* 1995), while the FcγRIIA has ITAM (Van de Winkel and Capel 1993). An additional level of complexity in the *FCGR2B* locus is that three alternatively spliced transcripts can be expressed: IIB1 and IIB2 differ by an insert of 19 amino acids in the FcγRIIB cytoplasmic domain, whereas the IIB3 form lacks part of the signaling sequence. FcγRIIB1 is expressed on B cells as the only currently recognized Fcγ receptor on B cells, whereas FcγRIIB2 is found on myeloid cells together with FcγRIIA (Li *et al.,* 2009). The *FCGR2C* gene has a stop codon (STP)/glutamine (Q) polymorphism at amino-acid position 13 in the first extracellular domain and has been described as an expressed protein on NK cells only when the 13Q allele is present (Ernst *et al.,* 2002; Breunis *et al.,* 2008). The *FCGR2C* gene encodes an extracellular domain that is highly homologous to FcγRIIB and an intracellular cytoplasmic domain that is nearly identical to the cytoplasmic domain of FcγRIIA. Characterization of the *FCGR2C* locus has suggested that the *FCGR2C* gene is the result of an unequal crossover event between *FCGR2A* and *FCGR2B* (Li *et al.,* 2009).

#### 5.9.1.2.3 FcγRIII (CD16)

Members of the FcγRIII (CD16) subfamily, including FcγRIIIA and FcγRIIIB, are encoded by two genes (*FCGR3A* and *FCGR3B* respectively) in humans (Table 4.0).

The FcγRIII is considered a low affinity receptor and it binds IgG molecules with similar affinity to that of FcγRII (Ravetch and Bolland 2001). However, FcγRIIIA binds monomeric IgG with an intermediate affinity and both FcγRIIIA and FcγRIIIB bind multimeric IgG and immune complex (IC) efficiently (Wu *et al.*, 1997; Koene *et al.*, 1997). FcγRIIIA ligand binding is further influenced by receptor glycosylation and is expressed as a transmembrane protein on monocytes, tissue-specific macrophages, DCs, γ/δT cells and NK cells (Nimmerjahn and Ravetch 2005). The stable expression of the protein on the cell surface as well as signal transduction through the FcγRIIIA receptor complex requires the γ-chain (or the T-cell receptor ζ-chain on NK cells) (Table 5.0). The *FCGR3B* gene is unique among the FcγR genes in that it does not encode a transmembrane portion; instead it encodes a glycosylphosphatidylinositol-anchored (GPI) receptor, FcγRIIIB and is expressed on the surface of neutrophils (where it is highly expressed, with 150,000–200,000 receptors expressed per cell) and on basophils (Li *et al.*, 2009).

**Table 5.0 Leukocyte FcγR subclass distribution, signaling properties, receptor heterogeneity and ligand specificity**

| | FcγRI | FcγRII | | | FcγRIII | |
|---|---|---|---|---|---|---|
| Receptor class | FcγRIA | FcγRIIA | FcγRIIB | FcγRIIC | FcγRIIIA | FcγRIIIB |
| Distribution | Myeloid cells Neutrophils (G-CSF and IFNγ-induced) | Myeloid cells, platelets, T cells (subset), endothelical cells | B cells, monocytes, macrophages | NK cells | Macrophages, monocytes (subset), NK cells, T cells (subset) | Neutrophils, eosinophils (IFNγ-induced) |
| Signaling motif (α-chain) | | ITAM | ITIM | ITAM | | |
| Accessory signaling subunit | γ | Γ | | ND | γ, ζ, β | CR3, FcγIIA |
| Allotype | | R131/H131 | I232/T232 | | V158/F158 | NA1/NA2 |
| hIgG subclass specificity | 3>1>4>>>2 | R131: 3>1>>>2,4; H131: 3>1,2>>>4 | 3>1>4>>2 | ND | 1=3>>>2,4[a] | 1=3>>>2,4[b] |

*ND=not determined [a]FcγRIIIA-V158 has a higher affinity for hIgG1 and 3 than FcγRIIIA-F158, and binds IgG4 [b]FcγRIIIB-NA1 has a higher affinity for hIgG3 than FcγRIIIB-NA2, and hIgG1- or hIgG3-opsonized particles are more efficiently ingested upon interaction with former allotype.* (Van Sorge *et al.*, 2003)

#### 5.9.1.2.4 FcγR Function

All FcγRs bear a unique ligand binding α-chain for cross-linking with ICs. However, in order to induce signaling, most rely on a rather promiscuous subunit such as the FcR γ- or ζ signaling chains of FcγRI and FcγRIIIA. Only the FcγRII class contains signaling motifs in its cytoplasmic tail, and does not require the

presence of a separate signaling subunit for initiation of leukocyte responses (Van Sorge *et al.,* 2003). On cross-linking, activating FcγRs initiate a signaling cascade which starts with the sequential activation of protein tyrosine kinases of the Src and Syk families resulting in the phosphorylation of the immunoreceptor tyrosine-based activation motifs (ITAM) in either the α-chain cytoplasmic tail, or in the associated signaling subunit (Ghazizadeh *et al.,* 1994; 1995).



Qualitatively, the ITAM of FcγRIIA, FcγRIIC and the FcR γ-chain trigger different effector functions due to differences in structure (van den Herik-Oudijk *et al.,* 1995). The FcγRIIB is the only FcγR that contains an immunoreceptor tyrosine based inhibition motif (ITIM) in its cytoplasmic tail and co-ligation of FcγRIIB with ITAM-containing receptors results in inhibition of ITAM-mediated effects, through activation of the SH2-containing phosphatase SHIP (Ono *et al.,* 1997; Lesourne *et al.,* 2001). FcγRIIB is expressed by B cells, and has also been recently detected in macrophages and monocytes (Tridandapani *et al.,* 2002). Co-ligation of the B cell receptor (containing ITAM) and FcγRIIB by immune complexes results in down-modulation of antibody responses both *in vitro* and *in vivo* and is capable of exerting inhibitory effects on monocyte/macrophage effector functions as well (Van Sorge *et al.,* 2003).

The FcγRIIIB is anchored into the cell membrane by a glycosyl phosphotidylinositol (GPI) unit and has no intrinsic signaling capacity as it lacks a

cytoplasmic tail, and does not associate with signaling subunits. FcγRIIIB mediated leukocyte effector functions such as antibody-dependent cellular cytotoxicity (ADCC) of red blood cells and phagocytosis by neutrophils is thought to be possible through (i) signaling induced by interaction with FcγRIIA, (ii) complement receptor 3 (CR3) serving as a signaling partner for FcγRIIIB (Van Der Pol and Van De Winkel 1998); or (iii) localization of FcγRIIIB in specialized signaling plateaux in the membrane ('rafts') to facilitate signaling since rafts are enriched in accessory signaling molecules, like Src protein kinases. Moreover, it's been shown that both FcγRIIA and FcγRIIIB move to rafts upon crosslinking, thereby enabling efficient interaction with signaling molecules (Zhou *et al.,* 1995). In antigen-presenting cells, such as macrophages and dendritic cells (DCs), activating FcγRs mediate cell functions such as phagocytosis, respiratory burst and cytokine production (TNF-α and IL-6); in neutrophils and NK cells, they trigger antibody-dependent cellular cytotoxicity and degranulation; and in mast cells, they induce degranulation (Kimberly *et al.,* 2002). Structural differences in FcγRs due to variation in glycosylation patterns also influence receptor function such as differences in affinity for IgG binding. High-mannose and complex-type oligosaccharide glycosylation of FcγRIIIA increases its affinity for monomeric IgG binding on NK cells than on monocytes (Edberg and Kimberly 1997). Interestingly, FcγRIIIB also exhibits variation in glycosylation pattern but this is due to a genetic polymorphism and not cell-type specific variability. As a result FcγRIIIB binding capacity for IgG1 and IgG3 displays allelic variation (Van Sorge *et al.,* 2003). FcγRIIA is unique in its expression on platelets, and cross-linking of FcγRIIA can directly activate platelets

resulting in platelet aggregation and granule release (Li *et al.,* 2009). FcγRI is capable of facilitating both MHC class I and class II-restricted antigen presentation with 100-fold less antigen than non FcγRI-mediated antigen presentation, and may induce a potent 'vaccine effect', as has been shown by the enhanced humoral response against FcγRI-targeted antigens (Van Der Pol and Van De Winkel 1998). FcγRIIA is less efficient in facilitating antigen presentation (Fanger *et al.,* 1997), but potently induces functions such as phagocytosis and degranulation. FcγRIIA is the only FcγR that interacts with IgG2, and is thought to be crucial for clearance of encapsulated bacteria, as IgG2 represents the main antibody subclass directed against bacterial polysaccharides (Van Der Pol and Van De Winkel 1998).

#### 5.9.1.3 FcγR Polymorphisms and Malaria

The *FCGR2A* displays a G to A point mutation (rs1801274) in the region specifying its ligand binding domain, which causes an arginine (R) to histidine (H) amino acid substitution at position 131. The FcγRIIA-H131 allotype displays higher binding efficiency for human IgG2 and IgG3, compared to FcγRIIA-R131 which binds weakly (Warmerdam *et al.,* 1991; Parren *et al.,* 1992). More importantly, FcγRIIA-H131 represents the sole receptor capable of efficient interaction with human IgG2. The capacity to effectively bind human IgG2 is therefore dependent on the individual's FcγRIIA genotype, and is subject to ethnic variation (Lehrnbecher *et al.,* 1999; Israelsson *et al.,* 2008). The FcγRIIA-131R/R genotype has been associated with protection against malaria, and the FcγRIIA-131H/H genotype with

susceptibility to the disease in some studies (Braga *et al.,* 2005). However, recent publications have implicated the FcγRIIA-131H allele instead to be associated to protection against severe malaria (Nasr *et al.,* 2007; Sinha *et al.,* 2008). This discrepancy is still not understood; and it's been suggested that differences in genetic backgrounds and/or differences in the pathogen pressure could be contributing factors (Israelsson *et al.,* 2008).



The *FCGR3A* gene displays a T to G substitution (rs396991) at nucleotide 559, resulting in a valine (V) to phenylalanine (F) substitution at amino acid position 176 – 176F/V (also referred to as 158F/V by counting from the N-terminus of the mature protein after cleavage) of the signal peptide which influences the receptor binding to IgG1, IgG3 and IgG4. FcγRIIIA-V/V158 has higher affinity for IgG1 and IgG3 than FcγRIIIA-F/F158 and a stronger inducer of IgG-mediated NK cell activity (Wu *et al.,* 1997; Koene *et al.,* 1997). The cytophyllic antibodies IgG1 and IgG3 have been shown to be associated with lower *P. falciparum* parasiteamia (Aribot *et al.,* 1996) or lower risk of clinical malaria (Taylor *et al.,* 1998). However, it has not been explained how this polymorphism might influence the outcome of *P. falciparum* malaria pathogenesis.

FcγRIIIB bears the neutrophil antigen (NA) polymorphism in its membrane-distal Ig-like domain and is found in three polymorphic forms, called human neutrophil antigen (HNA)-1a (formerly NA1), HNA-1b (formerly NA2) and HNA-1c

(formerly SH), which are encoded by *FCGR3B*1*, *FCGR3B*2* and *FCGR3B*3* genes, respectively (Bux *et al.,* 1999). The *FCGR3B*1* and *FCGR3B*2* alleles differ in five nucleotide positions (nucleotides 141, 147, 227, 277 and 349) (Najera 1989) which results in differences in receptor glycosylation (Huizinga *et al.,* 1990). FcγRIIIB-NA1 induces phagocytosis of IgG1- and IgG3-opsonized particles and binds immune complexed IgG3 more efficiently than the FcγRIIIB-NA2 allotype (Kimberly *et al.,* 1995). The nucleotide sequence of the FcγRIIIB coding region of *FCGR3B*3* is identical to the *FCGR3B*2* sequence except for a C→A single nucleotide exchange at position 266 leading to the expression of the FcγRIIIB-SH allo-antigen (Tong *et al.,* 2003; Van Sorge *et al.,* 2003) which has been associated with neonatal neutropenia (Van Der Pol and Van De Winkel 1998). It is not very clear how this allotype influences receptor function even though it has been associated with about a third of FcγRIIIB allele and significantly higher FcγRIIIB expression levels (Koene *et al.,* 1998). Some individuals, whose neutrophils lacked the FcγRIIIB molecule, and the corresponding gene, have been described in literature and are referred to as the $NA_{null}$ (De Haas *et al.,* 1995). A study in Thailand found no individual association of both FcγRIIA-131H/R and FcγRIIIB-NA1/NA2 polymorphisms with malaria severity but reported a combination of the FcγRIIIB-NA2 allele and the FcγRIIA-131H/H genotype to be associated with susceptibility to cerebral malaria. Thus, suggesting that FcγRIIA-131H/R and FcγRIIIB-NA1/NA2 polymorphisms have an interactive effect on host defence against malaria infection (Omi *et al.,* 2002).

A single T to C nucleotide change specifying an isoleucine (I) or threonine (T) at position 232 (rs1050501) of the FcγRIIB transmembrane region has been described (Kyogoku *et al.,* 2002). This single amino-acid substitution has been shown to affect the inhibitory function of FcγRIIB on B cells. The 232T allele is excluded from lipid rafts and has a decreased inhibitory potential toward B-cell receptor (Ono *et al.,* 1997) signaling (Floto *et al.,* 2005; Kono *et al.,* 2005) and has been associated with systemic lupus erythematosus in Asian populations, including Japanese, Chinese and Thais, (Kyogoku *et al.,* 2002; Siriboonrit *et al.,* 2003; Chu *et al.,* 2004) but not in African-American or Caucasian in the United States or Europe (Li *et al.,* 2003; Magnusson *et al.,* 2004). Su and others (Su *et al.,* 2004) identified a promoter haplotype that alters *FCGR2B* promoter activity. The less frequent promoter haplotype (-386C;-120A) showed increased promoter activity and drove higher receptor expression than the more frequent haplotype (-386G;-120T) (Su *et al.,* 2004). The implication of these receptor polymorphisms in malaria pathogenesis has yet to be reported.

### 5.9.2 IgG3 hinge region polymorphisms

It has been shown that the middle part of the heavy chain of IgG3 (hinge region) which covalently links the two γ3 chains to each other, is about 4 times larger than the same region in the three other human IgG subclasses (Michaelsen *et al.,* 1977). The superiority of IgG3 over IgG1 in parasite clearance is thought to be likely related to the length of its hinge region, which allows for

increased flexibility and ability to link both antigens and FcγRs (Redpath *et al.*, 1998). The unusually long hinge region of IgG3 has been postulated to be the result a quadruplication of a 45-nucleotide DNA segment resulting in a γ3 hinge region which is 62 amino acid residues long and consists of a 17-residue $NH_2$-terminal segment followed by a 15-residue segment which is identically and consecutively repeated three times (Michaelsen *et al.*, 1977). Furthermore, it has been found that the $NH_2$-terminal 17-residue segment shows about 70% homology 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. This observation led to conclusion that since $NH_2$-terminal 17-residue repetition shows 60 to 70% homology with the hinge of the other IgG subclasses, it may represent the primitive IgG hinge (*ibid*). Polymorphisms in the IgG3 hinge region which results in different hinge region lengths of IgG3 has been found and appears to influence antigen specific IgG3 levels (Theisen, unpublished data) implying the polymorphs might be relevant in malaria immunity. However, the consequence of the different hinge region allotypes in clinical malaria has not been studied.

### 5.9.3 Polymorphisms of erythrocyte surface molecules

In order to initiate the erythrocytic phase of its developmental cycle, the malaria parasite binds to erythrocyte surface molecules as the first stage in a complex series of events (which are still poorly understood) that ensures the parasite gets into the erythrocyte without destroying it (Sibley 2004). Once inside the erythrocyte, the

parasite manufactures a set of proteins that it sends to the cell surface (Kyes *et al.,* 2001). Some of these parasite-derived erythrocyte-membrane proteins bind to endothelial adhesion molecules and thereby cause parasitized erythrocytes to sequester in small blood vessels; an immune evasion strategy which prevents the parasites from circulating through the spleen and getting destroyed. Furthermore, since these parasite-derived molecules on the erythrocyte surface are themselves targets for immunological attack, the parasite has developed an extraordinary capacity for antigenic variation to evade the immune system (Kwiatkowski 2005).

The Duffy antigen (a primary invasion ligand for *P. vivax*), encoded by the *FY* gene, is a chemokine receptor that is expressed in various cell types. The Duffy antigen is expressed in erythrocytes in most populations but not in sub-Saharan Africa. A single nucleotide polymorphism (SNP) in the promoter of the Duffy antigen/chemokine receptor (DARC) gene alters the binding of the haematopoietic cell specific transcription factor GATA-1, thus inhibiting DARC expression (Miller *et al.,* 1975). The absence of this receptor from the red blood cell surface results in complete protection from *P. vivax* infection and this variant has reached fixation in most sub-Saharan Africans (Tournamille *et al.,* 1995). This completely prevents *P. vivax* from invading erythrocytes, and it accounts for the remarkable absence of *P. vivax* in sub-Saharan which has other species of malaria parasite to be extremely common (Miller *et al.,* 1976). It was this genetic discovery that led to the discovery of the *P. vivax* Duffy-binding protein, a parasite molecule that is critical for erythrocyte invasion by *P. vivax* (Chitnis and Miller 1994) and is now undergoing

clinical trials as a candidate agent for a vaccine against this species of parasite (Yazdani *et al.,* 2004).

In contrast to *P. vivax* however, *P. falciparum* appears to be quite liberal in its invasion pathways employing multiple ligands with considerable redundancy and a lot of research have focused on *GYPA*, *GYPB*, and *GYPC*, the genes encoding glycophorins A, B, and C, respectively (Hadley *et al.*, 1987). Various blood groups are determined by the erythrocyte- membrane sialoglycoproteins glycophorin A and B, and genetic deficiency of glycophorin A or B expression makes erythrocytes relatively resistant to invasion by *P. falciparum* (Facer 1983). Specific sialic-acid residues on the glycophorin A molecule are recognized by a Duffy-binding– like domain of *P. falciparum* erythrocyte-binding antigen 175 (*Pf*EBA175) (Orlandi *et al.,* 1992; Mayor *et al.,* 2005). On the other hand, glycophorin C is a minor component of the erythrocyte membrane that serves as a receptor for the *P. falciparum* erythrocyte-binding antigen 140 (*Pf*EBA140). In the coastal areas of Papua New Guinea where the Gerbich-negative blood group, (caused by a deletion of exon 3 of *GYPC*) is common, studies have shown a reduced erythrocyte invasion by *P. falciparum* (Maier *et al.,* 2003). Epidemiological studies indicate that this *GYPC* deletion does not alter the prevalence or density of asymptomatic malaria infection, but so far there has been no study of how the Gerbich-negative blood group affects the clinical severity of infection (Patel *et al.,* 2001; 2004).

Another erythrocyte-membrane protein that has been implicated in malaria resistance is an anion exchanger known as "band 3 protein," encoded by *SLC4A1*. A mutation resulting in a 27-bp deletion in this gene causes a form of ovalocytosis that is common in parts of Southeast Asia and appears to be protective both against malaria infection (Cattani *et al.*, 1987; Foo *et al.*, 1992) and against cerebral malaria (Genton *et al.*, 1995; Allen *et al.*, 1999). Though the mechanism of protection is not yet known it is thought to relate to the involvement of band 3 protein in endothelial cytoadherence or to some inhibitory effect on parasite invasion or growth (Kwiatkowski 2005).



### 5.9.4 Polymorphisms in Globin Genes

Hemoglobin which comprise the major component of erythrocytes is a tetrameric protein made of two $\alpha$ globin (encobed by the identical *HBA1* and *HBA2* genes) and two $\beta$ globin (encoded by the *HBB* gene) chains. The malaria parasite's developmental cycle depends heavily on a hemoglobin rich environment for its success. Thus an alteration in hemoglobin may affect the biochemical and cellular machinery of parasite development, and they may affect the ability of the spleen and other immune mechanisms to recognize parasites, by affecting the morphology, mechanical properties, or surface structure of the parasitized erythrocyte (Kwiatkowski 2005). *HBB* has three different coding SNPs each of which confers resistance against malaria and that have risen to high frequencies in different populations. The HbS (Penman *et al.*, 2009) allele is a glutamic acid-to-valine substitution at codon 6 (*Glu6Val)* of the $\beta$ globin chain, HbC ('hemoglobin C') is a

glutamic acid-to-lysine substitution at codon 6 (*Glu6Lys)*, and HbE('hemoglobin E') allele is a glutamic acid-to-lysine substitution at codon 26 *(Glu26Lys)* (Kwiatkowski 2005).

#### 5.9.4.1 Hemoglobin S

The HbS allele is found across large parts of sub - Saharan Africa as well as parts of the Middle East and was the first of the structural hemoglobin variant to be associated with malaria protection (PAULING *et al.,* 1949). The HbS homozygotes individuals suffer sickle-cell disease, but heterozygotes (HbAS) is associated with approximately a 2 fold reduced risk of malaria-fever and a 10 fold reduction in the risk of severe malaria (Gilles *et al.,* 1967; Hill *et al.,* 1991; Stirnadel *et al.,* 1999; Sokhna *et al.,* 2000; Ackerman *et al.,* 2005). Furthermore, it's been reported that when HbAS children suffer clinical malaria, the parasite densities are maintained at significantly lower levels than their non-HbAS counterparts (Williams *et al.,* 2005b). The exact mechanism by which HbAS confers protection against malaria is still not very well understood but presently there are two schools of thought which are not mutually exclusive: (1) suppression of parasite growth in red cells (Pasvol *et al.,* 1978) and (2) enhanced splenic clearance of parasitized erythrocytes (Shear *et al.,* 1993). Even though the major components being implicated are the innate mechanisms, it is also thought that some acquired immunological components might play very important roles and some recent studies support this view. First, a cohort study conducted in Kenya, reported that the protective effect of HbAS against mild malaria was found to increase from 20% to 56% in children between the ages of 2

and 10 years, suggesting that HbAS probably acts in synergy with or accelerates the acquisition of malaria – specific immunity (Williams *et al.,* 2005b; 2005c). In a second study conducted in Gabon, a FACS-based assay was used to show that, compared to normal children, HbAS subjects had significantly higher titers of IgG antibodies to the *P. falciparum* erythrocytes membrane protein 1 (*Pf*EMP1) (Marsh *et al.,* 1989; Cabrera *et al.,* 2005). Similarly, HbAS may contribute to a range of immunological responses to other antigens in malaria endemic populations (Ntoumi *et al.,* 1997a; 1997b; 2002; 2005). Due to the heterozygous advantage conferred by the HbAS, the HbS polymorphism is found at allele frequencies of about 10% in many parts of Africa, despite the lethal consequences for homozygotes (Kwiatkowski 2005).



#### 5.9.4.2 Hemoglobin C

Hemoglobin C is found in several parts of West Africa, although less commonly than HbS. It results in a much less damaging clinical phenotype than sickle-cell disease: homozygotes have a relatively mild hemolytic anaemia, and heterozygotes do not experience a significant reduction in hemoglobin levels (Diallo *et al.,* 2004). Early studies of HbC in malaria pathogenesis were not particularly convincing (THOMPSON 1962; 1963; Gilles *et al.,* 1967; Ringelhann *et al.,* 1976), however, more recent studies have shown that both heterozygotes and homozygotes of HbC are protective against severe malaria (Agarwal *et al.,* 2000; Rihet *et al.,* 2004; Mockenhaupt *et al.,* 2004b), but the protective effect appears to be substantially greater in HbCC (>90%) compared to HbAC (30%) (Modiano *et al.,* 2001). For

several years, the preferred hypothesis regarding the protective mechanism of HbC was that it reduces the ability of *P. falciparum* parasites to grow and multiply in variant RBCs (Friedman *et al.,* 1979; Pasvol and Wilson 1982; Olson and Nagel 1986; Fairhurst *et al.,* 2005). However, this mechanism is not strongly supported by *in vivo* data, where in most studies; HbC has not been found to exert an effect on parasite densities (Modiano *et al.,* 2001; Rihet *et al.,* 2004; Mockenhaupt *et al.,* 2004a). A plausible alternative mechanism has now been proposed that the protective effect of HbC may operate by increasing immune clearance of infected erythrocytes. This is based on *in vitro* observations of reduced parasite cytoadherence, due to reduced expression of the major parasite encoded RBC adhesion protein PfEMP1, clustering of erythrocyte band 3 protein, and altered surface topography of the erythrocyte membrane in the presence of hemoglobin C (Fairhurst *et al.,* 2005; Arie *et al.,* 2005). Since cytoadherence of iRBC is a major player in the pathogenesis of severe malaria these observations may explain how HbC might influence the incidence of severe disease, yet no marked impact on less severe outcomes. On the other hand, HbC RBCs like many other abnormal RBCs are mostly prone to oxidative stress and it is also possible that these observations might represent some form of *in vitro* artefact (Duffy and Fried 2006).

#### 5.9.4.3 Hemoglobin E

Hemoglobin E (HbE) is common in Southeast Asia, with carrier rates of 50% in some places, and analysis of haplotype structure suggests that the mutation is relatively recent and has risen rapidly to the current allele frequency in less than

5000 years under positive selective pressure by malaria (Ohashi *et al.,* 2004). This conclusion is supported by a hospital-based study conducted in Thailand, which found that the presence of HbE trait (HbAE) was associated with reduced disease severity in adults admitted with acute *P. falciparum* malaria (Hutagalung *et al.,* 1999). Homozygotes generally have mild anaemia. Although it has not been epidemiologically proven that HbE protects against severe malaria, this is assumed to be the case, and it has been observed that erythrocytes from HbE-heterozygous individuals are relatively resistant to invasion by *P. falciparum* suggesting a possible protective mechanism for HbE (Chotivanich *et al.,* 2002).

**5.9.4.4 Alpha Thalassemia**

Over half a century ago, J. B. S. Haldane proposed balanced polymorphism as the explanation for why thalassemia had risen to high frequencies-approaching fixation-in certain populations (HALDANE 1949). According to him, heterozygotes might be protective against some important disease, with malaria being the obvious candidate since the global distribution of thalassemia encompasses the major malarious regions of Africa and Asia and Mediterranean regions where malaria is/or was once common. This concept has been given more credibility by some vital observations. For example, the Tharu people of Nepal have both a much higher allele frequency of α-thalassemia, of approximately 0.8, and a much lower incidence of malarial illness than do other ethnic groups that inhabit the same region of Nepal (Modiano *et al.,* 1999a). Arguably the strongest population-genetic evidence comes from a detailed survey in Melanesia that showed that the frequency of $\alpha^{+}$_

thalassemia varied according to both altitude and latitude in a manner that was highly correlated with malaria endemicity, whereas haplotypic analysis seemed to rule out the possibility that this could have arisen because of founder effects (Flint *et al.,* 1986). There is now consistent evidence from a number of population studies, showing that α-thalassemia is associated with protection from severe and fatal malaria. In Kenya, a study found both heterozygous and homozygous $\alpha^{+}$_ thalassemia to be protective against severe malaria in children (Williams *et al.,* 2005c), whereas a study of Ghanaian children associated heterozygotes with protection (Mockenhaupt *et al.,* 2004b). In Papua New Guinea, the risk of severe malaria was found to be reduced by 60% in children who were homozygous for $\alpha^{+}$_ thalassemia and to a lesser degree in heterozygotes, but this result did not seem to be malaria-specific, since a protective effect was also observed for other childhood infections as well (Allen *et al.,* 1997). The protective mechanism of thalassemia is not clearly understood. However, flow-cytometry studies *in vitro* have shown that erythrocytes with the $\alpha^{+}$_ thalassemia phenotype showed reduced parasite growth (Pattanapanyasat *et al.,* 1999) and increased binding of antibodies from malaria-immune sera (Williams *et al.,* 2002). Enhanced splenic clearance of malaria-infected cells is a further possibility but is difficult to test *in vivo*. Interestingly, however, unlike HbAS (Williams *et al.,* 2005a), α-thalassemia has no effect on parasite densities (Oppenheimer *et al.,* 1987; Williams *et al.,* 1996; Allen *et al.,* 1999; Mockenhaupt *et al.,* 2004b; Williams *et al.,* 2005c; Wambua *et al.,* 2006).

### 5.9.4.5 Beta Thalassemia

The beta (β) thalassemia trait has also been linked to protection against *P. falciparum* malaria (Weatherall 2001; Weatherall *et al.,* 2002) particularly in malaria endemic regions of the Mediterraneans, North Africa and Middle East, India, China, Southeast Asia (Willcox *et al.,* 1983a; Kazazian and Boehm 1988; Penman *et al.,* 2009). However, in sub-Saharan Africa, β-thalassemia is only found in very limited parts of West Africa such as Liberia where it co-exist with malaria (Willcox *et al.,* 1983b; Pasvol 2006). The β-thalassemic allele ($\beta^{++}$) found in this region is a clinically milder phenotype than those of the Mediterranean (Willcox *et al.,* 1983b). Beta (β)-thalassemia is an autosomal recessive disease characterized by hypochromic, hemolytic anaemia and dependence on blood transfusions to sustain life with homozygocity (β-thalassemia major) known to shorten life expectancy by 25 to 30 years on average (Kazazian and Boehm 1988). More than 70 different types of mutations in the β-globin gene have been identified to cause β-thalassemia, but each population seems to have its own characteristic set and frequency of β-thalassemia mutations (Lie-Injo *et al.,* 1989). The protective mechanism of β-thalassemia has still not satisfactorily been explained. One possible mechanism which might explain the mechanism of protection conferred by the β-thalassemia trait in *P. falciparum* malaria is non-iron heme-analog mediated inhibition of heme polymerization (Martiney *et al.,* 1996). *P. falciparum* trophozoites degrade hemoglobin in acidic food vacuoles to meet their amino acid requirements (Gluzman *et al.,* 1994; Olliaro and Goldberg 1995). In the process heme is released and converted to a non-toxic polymer (hemozoin or malaria pigment) by a parasite-

specific heme polymerizing activity (Slater 1992). Heme polymerization involves the cross-linking of the iron of one heme to a carboxylate group of another (Slater *et al.,* 1991; Slater 1992). A change in the central metal moiety could inhibit the polymerization process and lead to the accumulation of the toxic heme in the food vacuole of trophozoites thereby killing them (Martiney *et al.,* 1996). Zinc protoporphyrin IX (ZnPP) is a naturally occurring metalloporphyrin, used clinically to inhibit neonatal heme catabolism (Rodgers *et al.,* 1990). It has been shown that β-thalassemic intra-erythrocytic ZnPP levels are higher than in normal erythrocytes and this intra-erythrocytic ZnPP could gain access into the food vacuole, as a competitive substrate and thus inhibit heme polymerization activity (*ibid*). A more recent study (O'Donnell *et al.,* 2009); conducted in a low malarial transmission region of Sri Lanka found that HbE β-thalassemic individuals had significantly higher antibody titres to crude *P. vivax* antigens and *Pv*MSP1-19 but to a lesser extent for crude *P. falciparum* antigens than those of age-matched controls from the same region. This implied a possible role of these traits in the induction of stronger antibody response in vivax malaria. O'Donnell and colleagues explained this observation to mean that, it was due to the high susceptibility of HbE β-thalassemic individuals to malaria infection particularly of the *P. vivax* species (*ibid*). Their explanation was based on the observation that patients with HbE β-thalassemia have enormously expanded erythroid bone marrow, ineffective erythropoiesis, and shortened red-cell survival with significantly increased reticulocyte counts (Weatherall *et al.,* 2002). *P. vivax* is known to have a particularly strong liking for reticulocytes and young erythrocytes (Galinski *et al.,* 1992; Rodriguez *et al.,* 2002)

while *P. falciparum*, though can invade erythrocytes of all ages was also shown to have a predilection younger erythrocyte populations (Pasvol *et al.,* 1980). Thus, the elevated antibody response to *Pv*MSP1-19 could be as a result of sustained exposure to the parasites in the HbE β-thalassemic individuals. Several other mechanisms have also been proposed to explain the molecular basis of protection against malaria conferred by β-thalassemia. These include host erythrocyte biochemical abnormalities (Nagel and Roth, Jr. 1989), high hemoglobin F content (Brockelman *et al.,* 1987), iron deficiency (Nurse 1979), enhanced immune recognition (Luzzi *et al.,* 1991), impaired rosette formation (Carlson *et al.,* 1994), and enhanced phagocytosis on ring-parasitized mutant erythrocytes (Ayi *et al.,* 2004).

#### 5.9.4.6 Glucose – 6 – Phosphate Dehydrogenase (G6PD)

*Plasmodium* parasites growing in an infected RBC need to breakdown hemoglobin to meet their huge nutritional requirement and also to make room to grow. In the process, potentially toxic by-products such as iron are released that is a major source of oxidative stress. A defense mechanism against oxidative stress within the RBCs is production of the electron donor, nicotinamide adenine dinucleotide phosphate (NADPH) by the enzyme glucose-6-phosphate dehydrogenase (G6PD), encoded by the *G6PD* gene on chromosome X. There are many different variants of G6PD, and those that markedly compromise enzyme activity result in hemolytic anaemia. G6PD deficiency is the commonest enzymopathy in man affecting over 400 million persons worldwide (Beutler *et al.,* 1991; Ruwende *et al.,* 1995). The geographical

distribution of G6PD deficiency is consistent with evolutionary selection by malaria (Ganczakowski *et al.,* 1995), and analysis of haplotypic structure at the *G6PD* locus supports the hypothesis of recent positive selection (Tishkoff *et al.,* 2001; Sabeti *et al.*, 2002). In Africa, G6PD is essentially a triallelic polymorphism (G6PD B, G6PD A and G6PD A-) with varying enzyme activity. Some studies have shown that the heterozygosity (G6PDB/A–, G6PDA/A–) confers protection against both severe and mild malaria (Bienzle *et al.,* 1975; Hill *et al.,* 1991; Ruwende *et al.,* 1995) but this has not been confirmed by others (Lell *et al.,* 1999; Migot-Nabias *et al.,* 2000; Parikh *et al.,* 2004). Deficient G6PD enzyme activity has been shown to correlate with protection against severe malaria in Nigerian children (Gilles *et al.,* 1967) and a study of 12,000 Gambian and Kenyan children found that the common African form of G6PD deficiency (G6PD A-) is associated with approximately 50% reduced risk of severe malaria in female heterozygotes and in male hemizygotes (Ruwende *et al.,* 1995). The mechanism of protection is thought to be by a reduced parasite replication in G6PD-deficient erythrocytes (Luzzatto *et al.,* 1969), but the parasite appears to counter this by manufacturing G6PD itself (Usanga and Luzzatto 1985).

#### 5.9.4.7 Haptoglobin

Another protein of interest with regards to oxidative stress and malaria pathogenesis is haptoglobin, encoded by *HP* gene. Haptoglobin functions primarily as a free hemoglobin scavenger in the plasma thereby preventing hemoglobin induced - oxidative tissue damage but has also been shown to inhibit the development of

malaria parasites in vitro (Imrie *et al.*, 2004); and also appears to reduce parasite load, as determined by murine gene knockout studies (Hunt *et al.*, 2001). There are conflicting data on haptoglobin associations with the risk of malaria. Ahaptoglobinaemia (i.e. the absence of haptoglobin) is a common phenotype in malaria infected individuals, but this is not entirely dependent on genotype and can be affected by the disease status of the individual (Rougemont *et al.*, 1980; Boreham *et al.*, 1981; Hill *et al.*, 1987). Although studies of the relative frequencies of the protein isoforms of haptoglobin have shown an association with malaria infection, no evidence of this was seen in a more recent, larger study in West Africa (Elagib *et al.*, 1998; Quaye *et al.*, 2000). The haptoglobin 1-1 genotype, characterized by protein electrophoresis, has been associated with susceptibility to severe *P. falciparum* malaria (Elagib *et al.*, 1998; Quaye *et al.*, 2000), although a DNA-based study of haptoglobin polymorphisms in the Gambia failed to detect such an association (Aucan *et al.*, 2002)

# CHAPTER THREE

## 6.0 METHODOLOGY

### 6.1 Chemicals, Reagents and Equipments

The sources and manufacturers of reagents, buffers, solutions and equipments used in the study are shown in Appendix I.



### 6.2 Study Area

The study consisted of two community based cohort studies and a hospital based case-control study. The two cohort studies were conducted in Asutsuare (about 120km north-east of Accra) and six other surrounding villages: Volivo, Mafikorpe, Kewum-Atrobinya, Osuwem - Gbese, Osuwem-Lanor, and Avakpo of the Dangme West District of Accra, Ghana. The population is predominantly of the Dangme ethnic group but are interspersed with other ethnic groups such as the Ewe and Akan. They are essentially peasant communities with rice farming as one of the major means of subsistence. The villages are only about 5-8km apart and there are networks of canals which aid in irrigation of the rice farms when the rainfalls are low. Both children and adults work on the rice farms with the children mainly engaged in scarring birds away when the rice start to bear. Rainfall is usually highest from March to August and also in November and December just before the beginning of the harmattan or dry season. Thus, there are two seasonal peaks of malaria transmission in the area: from March to August and also in the months of November and December coinciding with the major and minor rainy seasons respectively. However, there is also a relatively minimal transmission throughout the

remaining times of the year, which is typical of the dry season. There are two health centres: the Osudoku Community Health Centre at Asutsuare and the Osuwem Community Health Centre at Osuwem-Gbese which serve all these communities. In addition, the Akuse Hospital about 10km away serve as a referral hospital for cases beyond the capacity of the health centres.

Archived samples from a previous hospital based study were also used in this study. The hospital based study was conducted at the Department of Child Health (DCH), Korle-Bu Teaching Hospital, Accra. The Korle-Bu Teaching Hospital has a referral unit for many paediatric cases including malaria which constitutes about 40% of all outpatient cases at the DCH, most of which are reported within the peak malaria season (between May and October). Fifty percent of malaria cases reported at DCH are severe and data from a previous study in 2000 at the hospital showed an overall mortality rate of 20%, 14% being cerebral malaria and 7% being severe malaria anaemia (SMA) (*Akanmori et al., unpublished*).

**6.3 Study Design**

The two cohort studies were both longitudinal involving a total 669 children ranging 1 to 12 years of age (cohort 1) and 550 individuals from 1 to 29 years of age (cohort 2), spanning 2 consecutive malaria transmission seasons. At baseline (enrolment), 5ml EDTA-anticoagulated venous blood, filter paper blood blot and thick and thin film blood slides were obtained from all individuals prior to the malaria transmission season (May-2008 for cohort 1 and February2009 for cohort 2) for baseline immunological

and parasitological determinations. During enrolment of individual into cohorts, blood group and sickle cell status were determined by the blood grouping kit and the sodium metabisulphite test respectively. The hemoglobin (Olson and Nagel 1986) level was also measured using the Hemocue - Hb 201 (Angelhom, Sweden) at both enrolments. The blood was centrifuged using the Forma 3L Gp 4500R centrifuge (Thermo electron corporation, MA, USA) to separate plasma and peripheral blood mononuclear cells (PBMCs) and stored at -80°C and in liquid nitrogen respectively. Thick and thin blood film slides were Giemsa stained and examined for asymptomatic parasitaemia. A standardised questionnaire was used to obtain relevant epidemiological and clinical data of each study participant at the enrolment of each cohort. Morbidity surveillance during the period of both cohorts was by both active and passive detection of mild clinical malaria. The active surveillance was through weekly visits to participants' homes, where a morbidity questionnaire (investigating symptoms occurring in the preceding week) was administered and the presence or absence of fever was ascertained (axillary temperature of ≥37.5°C, measured or reported) by trained Field Assistants. Participants found to be unwell were referred for free medical treatment at either the Osudoku Community Health Centre at Asutsuare or the Osuwem Community Health Centre which served as health support centres during the period of both cohorts and where they were also able to access on their own when required at any time during the study (passive case detection). In both the active and passive case detection, filter paper blood blot and thick and thin film blood slides were obtained from referred study participants with febrile temperature (>37.5°C) or reported febrile temperature prior to treatment. Clinical episodes of malaria were treated with

artesunate-amodiaquine combined dose therapy which was the recommended standard treatment for malaria in Ghana by the Ministry of Health at the time of the studies. The active and passive case detection morbidity survey (follow-up) lasted for 42 and 36 weeks for cohort 1 and 2 respectively. Every month (cohort 1) and every three (3) months (cohort 2) during the follow-up periods, a small amount of blood (about 500ul) was collected from finger prick for plasma for immunological analysis and buffy coats for host genetics analysis. In addition blood slides and filter paper blood blots were made for all participants to determine asymptomatic malaria parasitaemia. At the end of the each cohort and on the basis of the clinical and parasitological data obtained, the study populations were divided into: (1) those susceptible, in which parasitaemia was associated with febrile disease, and (2) those apparently protected against clinical manifestation despite parasitaemia/or without detectable parasitaemia by microscopy.

In hospital based case-control study, EDTA anti-coagulated blood was obtained from individuals aged 1 to 12 reporting for medical care at the DCH, Korle Bu from June to August 2003, whose parent or guardians consented to have them enrol in the study. Plasma and buffy coats were separated and stored for immunological and genetics analysis respectively. Thick and thin blood films were prepared for each participant and stained with Giemsa and examined for malaria parasites by light microscopy. Malaria parasites were counted against 200 WBCs and parasite densities estimated by multiplying the total WBC counts, obtained from a hematological analyser (Sysmex K21, Japan), by parasites/200 WBCs. The hematological parameters such as hemoglobin levels, total WBC counts, total RBC counts and MCV were

measured with an auto hematological analyser (Sysmex K21, Japan). Sickling test was done for each patient by using the sodium metabisulphite test and sickling-positive patients were excluded from the study.

### 6.4 Ethical Clearance

Ethical clearance for both cohorts as well as the hospital based case-control study were given by the Institutional Review Board (IRB) of the Noguchi Memorial Institute for Medical Research, University of Ghana; Legon - Ghana.

### 6.5 Case Definition

In both of the cohort studies and the hospital based case-control study, malaria was defined as reported or measured fever (>37.5 °C) plus parasitaemia ≥ 2500/µl and at least one other sign of malaria (vomiting, diarrhea, headache, etc).

#### 6.5.1 Inclusion Criteria

*Cohort studies:* All healthy individuals living in Asutsuare and its environs who gave their informed consent or whose parent or guardians consented to have them enrolled in the studies were included.

*Hospital based study:*

CM cases: This was defined as unconsciousness, with Blantyre coma scale score of < 3 for duration of > 60 minutes. The patient must have no record of recent

severe head trauma and other cause of coma or neurological diseases. Any hemoglobin value was included.

SMA cases: This was defined as haemoglobin < 5g/dl, fully conscious, no cases of severe bleeding reported or observed convulsion.

### 6.5.2 Exclusion Criteria

A child was not allowed to participate in the study if a parent or guardian refused to sign the informed consent form and also adults who did not give their informed consent were not included in the study. Also a participant with confirmed malaria based on clinical and parasitological examinations at baseline sampling was excluded from the final analysis of the data.

## 6.6 Laboratory Measurements

### 6.6.1 Parasitological Examination

Light microscopy was used to examine and quantify malaria parasites against 200 WBC. The parasite density was then calculated using the formula

Parasite density = (Count x 8000)/200

Where ‘count’ is number of asexual blood stage parasite counted and 8000 and 200 are constants representing number of RBCs and WBCs respectively.

### 6.6.2 Enzyme-Linked Immunosorbent Assays (ELISAs)

#### 6.6.2.1 Malaria Antigens

##### 6.6.2.1.1 Recombinant Antigens

The malaria antigens used in this study included a recombinant GLURP protein R0 containing the conserved non-repeat N-terminal region, (amino acids 25–514), and GLURP R2 (amino acids 705–1178) of the carboxy-terminal repeat region all expressed in *Escherichia coli* (Theisen *et al.*, 1995) (supplied by Dr. Michael Theisen from State Serum Institute, Copenhagen). The *Pf*AMA1 antigen is the ectodomain (N terminal) portion of the antigen from the FVO strain of *P. falciparum*. It comprises amino acids 25 - 545 and was expressed as a recombinant antigen in the methylotrophic yeast *Pichia pastoris* (supplied by Edmond Remarque of BPRC, Netherlands). The EBA-175 RII containing the amino acid residues 144 to 753 and expressed in yeast *Pichia pastoris*, (donated by Dr. Annie Mo of Rockville USA). The recombinant MSP-3 of the FVO strain expressed in *E. coli*, (supplied by Dr. Richard Shimp from NIAID, NIH, USA).

##### 6.6.2.1.2 The MSP-1 Hybrid Antigen

The MSP-1 hybrid is a synthetic gene designed to incorporate sequences derived from all three serotypes of the highly polymorphic Block 2 of the Merozoite Surface Protein 1 (MSP-1). One version of the semi-conserved Block 1 was added to the N-terminal portion to form a construct of 348 amino acids in length which was then optimized for codon usage in *E. coli* to maximize expression. The upstream

sequence corresponds to the Block 1 sequence from K1-type MSP-1. This is followed by artificially designed sequences which include the 3 main variants of the Block 2 found in over 150 laboratories and field isolates. The one type (RO33 type), contains minimal point mutations only. The other 2 types (K1 type and MAD20 type) are characterized by unique, serotype-specific flanking sequences, each containing different sets of tripeptide repeats, which differ within each serotype by both order and number.


**Figure 3.0** MSP-1 hybrid gene construct

The resulting MSP-1 Block 2 hybrid construct (in a pET24a expression vector) was expressed as a 31kDa untagged protein in the *E.coli* BLR (DE3) pLysS strain (supplied by Dr. David Cavangh of University of Edinburgh).

#### 6.6.2.1.3 Synthetic Peptides Antigens

The synthetic peptides; LR146 - (IIDIKKHLEKLKIEIKEKKEDLENL) and AS202.11 - (QLEEKTKQYNDLQNNMKTIKEQNEHLKNKFQSMGK) used in the study (obtained from Dr. Giampietro Corradin of the Biochemistry Department of the University of Lausanne, Switzerland). A detailed description of the peptide synthesis has been published elsewhere (Villard *et al.,* 2007). Briefly, peptides were synthesized on the Applied Biosystem synthesizer 431A and 433A (Foster City, CA) using solid-

phase Fmoc chemistry. Crude peptides were purified by RP-HPLC (C18 preparative column) and analyzed by mass spectrometry (MALDI-TOF; Applied Biosystem, Foster City, CA).

### 6.6.2.2 ELISA Procedure

Isotype IgM and IgG as well as $IgG_{(1-4)}$ subclass levels to the malarial recombinant antigens GLURP R0, GLURP R2, MSP1 hybrid, EBA 175, AMA1 FVO, MSP3 FVO, and the peptides LR146 and AS202.11 were measured by indirect ELISA using a modified version of the Afro Immuno Assay (AIA) ELISA protocol described elsewhere (Lusingu *et al.*, 2005; Sirima *et al.*, 2007; Nebie *et al.*, 2008; Dodoo *et al.*, 2008).Briefly, 100µl of antigens was coated directly to each well in a 96-well microtitre ELISA plate (Maxisorp Nunc, Denmark) in coating buffer (plain PBS, pH 7.04) at 1.0µg/ml for the recombinants and 5.0µg/ml for the peptides. All antigens tested were optimized and shown to be stable for at least three weeks, when antigen-coated plates and plasma/serum dilutions are refrigerated (2 to 8°C). Coated plates were kept in a refrigerator at 2 to 8°C overnight. Plates were then washed four times in washing buffer (PBS with 0.1% Tween-20 and 0.5 M NaCl) with 30 seconds incubation between each wash 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 blocking buffer (PBS with 5 % milkpowder, 0.1% Tween-20) and incubated at room temperature in a humidified chamber for 1 hour. Plates were then washed four times in washing buffer and

serum/plasma samples diluted at 1:200 in serum dilution buffer (PBS with 2.5 % milkpowder, 0.1% Tween-20 and 0.02% Na-azide) was added at 100µl/well in duplicates. To control for inter-assay and day-to-day variations in the standardized ELISA procedure, each assay (ELISA plate) includes a calibration curve obtained by a 2-fold titration of pool of hyper immune sera/plasma known to be positive for the antibodies (IgG, IgM and IgG1 to IgG4) to the specific malaria antigens tested. In addition, each plate also included a negative control sample (a pool of plasma sample from Danish blood donors never exposed to malaria), 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) which served as internal controls to allow for detection of a failed assay run. The plates with the samples were then incubated at room temperature for 2 hours in a humidified chamber after which they were washed four times in washing buffer and the appropriate secondary (detection) antibody for the specific antibody to be determined added at 100µl/well. The optimised dilutions for the detection antibodies used in the assays were; goat anti-human IgG (γ) HRPO conjugated (Invitrogen Corporation, Camarillo, CA; USA) (1:80000) and goat anti-human IgM (µ) HRPO conjugated (Invitrogen Corporation, Camarillo, CA; USA) (1:3000) for the isotypes. 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. The detection antibodies for both isotype IgG and IgM as well as IgG1-4 subclasses were all selected on the basis of low cross reactivities among themselves. The plates with the conjugates were then incubated for 1 hour at room temperature in a

humidified chamber after which they were washed four times in wash buffer and padded dry. Bound secondary antibody was quantified by colouring with ready to use TMB (3, 3', 5, 5'-Tetramethylbenzidine) substrate (Kem-En-Tec Diagnosis A/S, Taastrup, Denmark) and incubated in the dark for 30minutes. Optical density (OD) was read at 450 nm with a reference at 620nm in the Biotek EL 808 ELISA plate reader (Biotek Instruments, Winooski, VT; USA). Optical density (OD) 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). Samples were re-tested if the coefficient of variation between duplicate absorbance values were higher than 15% and plates were also re-tested if the R-square value of the standard curve was less than 97%.

### 6.6.3 Genetic Analysis

#### 6.6.3.1 Genomic DNA Purification and Amplification

Human genomic DNA was purified from buffy coat samples using the Promega Maxwell® 16 DNA purification kit and the concentration of DNA obtained for each sample determined with the NanoDrop® (ND-1000, NanoDrop Technologies, USA). Each sample was then diluted with PCR-quality grade water to about 5ng/µl and Whole Genome Amplification (WGA) was performed on each sample to increase the available DNA using the AmpliQ Whole Genome Amplification Kit according to manufacturer's instruction. Following the WGA, each sample was diluted (1:50) to a concentration of about 20 – 40ng/µl and stored at -20°C until required for genotyping.

#### 6.6.3.2 Genotyping of FcγR2A-131H/R (rs1801274) polymorphism

##### 6.6.3.2.1 PCR Primers

The FcγR2A - 131H/R ligand binding polymorphism was genotyped using the allele-specific restriction enzyme digestion as described by Jiang and colleagues with slight modifications (Jiang *et al.,* 1996). The sense primer 5'-GGAAAATCCCAGAAATTCTC**G**C-3' (DNA Technology A/S, Risskov, Denmark) used starts from the exon which encodes the second extracellular domain upstream of codon 131 and ends immediately 5' to the polymorphic site. One nucleotide substitution (G – shown in bold) was made in order to introduce a *Bst*UI site (5' – CGCG – 3') into the PCR product when the next nucleotide is G but not when it is A. The antisense 5'- CAACAGCCTGACTACCTATTACG**CG**GG-3'

(DNA Technology A/S, Risskov, Denmark) was placed in the downstream introns and contains two nucleotide substitutions (CG – shown in bold) which introduces an obligate *Bst*UI site into all the resulting PCR products which was used as an internal control for successful *Bst*UI digestion. These primers were carefully chosen to specifically amplify only the *FCGR2A* gene and not the highly homologous *FCGR2B* and *FCGR2C* genes. To ensure this, the antisense primer compared to the corresponding sequence of the *FCGR2B* and *FCGR2C* (which are identical to each other in this region) has six mismatches and one gap.



#### 6.6.3.2.2 PCR Amplification

The PCR amplification was performed in a 50µl reaction containing 40 – 80ng genomic DNA, 20mM of each primer (DNA Technology A/S, Risskov, Denmark), 2.5mM of each of the four deoxynucleotide triphosphates (dNTPs), 2.0 units of TEMPase Hot Start DNA Polymerase (Ampliqon, BIOMOL GmbH, Hamburg, Germany) and 125mM $MgCl_2$ (Ampliqon), and 10X TEMPase buffer I (Ampliqon) containing 15mM $MgCl_2$. The cycling conditions were: initial denaturation at 95 °C for 15 min, 35 cycles of denaturation at 94 °C for 30 sec, annealing at 60 °C for 30 sec and extension at 72 °C for 45 sec, followed by a final extension at 72 °C for 10 min in a PTC – 200 thermal cycler (M.J Research Inc; Massachusettes, USA). The PCR product was analysed by electrophoresis on 3% (w/v) agarose (SeaKem®GTG® Agarose, Lonza, Rockland, ME, USA) with ethidium bromide (AppliChem, Damstadt, Germany) staining and pictures taken using the Ultra-Violet Product

(UVP) Biospectrum AC Imaging system with Biochemi-camera (AH Diagnostics, Cambridge, UK). The result was a 366 bp product (Appendix II) which contained an introduced *Bst*UI site in the 3' region and a potential *Bst*UI site in the 5' region depending on the polymorphism of the gene.

#### 6.6.3.2.3 Restriction enzyme digestion of the PCR product

The crude 366bp PCR product was then subjected to RFLP analysis by *Bst*UI (New England BioLabs® Inc., Ipswich, MA, USA) digestion in a 20µl reaction mixture containing 10 µl, of PCR product, 4units of *Bst*UI restriction enzyme and corresponding buffer (NE buffer 4) and incubated at 60°C for 2 hours in a PTC – 200 (M.J Research, Inc; Watertown, Massachusettes, USA). The final product was analysed by electrophoresis on 3% (w/v) agarose (SeaKem®GTG® Agarose, Lonza, Rockland, ME, USA) with ethidium bromide (AppliChem, Damstadt, Germany) staining and pictures taken. The product size observed for the various outcomes were: uncut/undigested PCR product = 366 bp; $H/H^{131}$ = 343 bp; $R/R^{131}$ = 322 bp; and $H/R^{131}$ = 343 bp and 322 bp using a 50bp molecular weight marker (Appendix IIa). To exclude restriction digest failure, undigested product was included on the gel with genotype assignment made on only those samples that showed complete digestion.

#### 6.6.3.3 Genotyping of FcγR2B-232I/T (rs1050501) polymorphism

The FcγR2B-232I/T SNP was genotyped by a three step approach. First, a long-range PCR method described by Su and colleagues was adopted (Su *et al.*, 2004) with minor modifications to specifically amplify the *FCGR2B* gene from genomic DNA using the *PfuUltra*® Hotstart High – Fidelity DNA Polymerase (Stratagene, USA) system. The sense primer (5'-CTCCACAGGTTACTCGTTTCTACCTTATCTTAC-3') (DNA Technology A/S, Risskov, Denmark) is non-specific to FcγR2B and anneals at both *FCGR2B/C* at –2-kb of their promoters whereas the antisense primer (5'-GCTTGCGTGGCCCCTGGTTCTCA-3') (DNA Technology A/S, Risskov, Denmark) anneals at the FcγRIIB - specific sequence in intron 6 between exons 6 and 7. The 25µl reaction mixture contained 40ng genomic DNA; 10mM of each primer, 0.125mM of each dNTPs, and 1.0 unit of *PfuUltra*® hotstart high-fidelity DNA polymerase with the corresponding 10X *PfuUltra* HF reaction buffer ( Stratagene, USA). The PCR conditions were 92°C activation for 2 min, 10 cycles of 92°C for 10 sec and 68°C for 30 min, followed by 20 more cycles with the extension time which increased by 10 sec per cycle in a 2720 Thermal Cycler (Applied Biosystems, Singapore). Secondly, the resulting ~15-kb product was then used as a template for the nested PCR to specifically amplify a 97bp fragment of the *FCGR2B* gene containing the 232I/T SNP with the sense primer (5'-TCACCGATGGGGATCATTGT – 3') (DNA Technology A/S, Risskov, Denmark) and antisense primer (5 – Biotin-TCCGCTTTTTCCTGCAGTAGA – 3') (DNA Technology A/S, Risskov, Denmark). The 50ul PCR reaction mixture contained 2µl

of the long-range PCR amplified DNA product, 20mM of each primer, 1 unit of TEMPase hotstart DNA polymerase with the corresponding 10X TEMpase buffer I (Ampliqon, BIOMOL GmbH, Hamburg, Germany), 2.5mM of each of the four dNTPs (Ampliqon, Germany). The cycling conditions were 95°C for 15 min, 30 cycles of 94°C for 30 s, 63°C for 30 s, and 72°C for 45 sec, and followed by a 10-min extension at 72°C in a PTC – 200 (M.J Research, Inc; Watertown, Massachusettes, USA). Finally, the nested-PCR product was genotyped by pyrosequencing using the Pyro Gold Reagents kit (Pyrosequencing AB, Uppsala, Sweden) on a PSQ$^{TM}$ 96 Systems (Biotage, Westborough, MA) following the manufacturer's instructions. The pyrosequencing primer was 5' – GGCTGTGGTCACTGG – 3' which gave pyrograms (Appendix IIc) distinctive of the polymorphism genotype present.

#### 6.6.3.4 Genotyping of FcγR3A-176F/V (158F/V) (rs396991) polymorphism

The FcγRIIIA genotype for each individual was determined in two separate allele-specific PCR reactions as has been described (Kobayashi *et al.,* 2007), with minor modifications. The FcγRIIIA-specific forward (5' – TCACATATTTACAGAATGGCAATGG - 3') was used in combination with either V - specific (nt 559 G - specific) (5' - TCTCTGAAGACACAT TTCTACTCCCTAC - 3') or F - specific (nt 559 T - specific) (5' - TCTCTGAAGACACATTTCTACTCCCTAA - 3') reverse primer (DNA Technology, A/S, Denmark) were used in separate reactions to genotype a single

individual since each forward – reverse primer pair gives the same product size of 138bp. To avoid false negatives, sense (5' – CAGTGCCTTCCCAACCATTCCCTTA - 3') and antisense (5' - ATCCACTCACGGATTTCTGTTGTGTTTC - 3') primers (DNA Technology, A/S, Denmark) specific for a 439bp fragment of the human growth hormone gene were added to each reaction to serve as internal control. Each 25µl reaction mixture contained, 40ng genomic DNA, 2 units of TEMPase hotstart DNA polymerase with the corresponding 10X TEMpase buffer I (Ampliqon, BIOMOL GmbH, Hamburg, Germany), 2.5mM of each of the four dNTPs and 62.5mM $MgCl_2$ (Ampliqon, Germany), 20mM each of FcγRIIIA-specific forward and either F or V – specific reverse primers together with 10mM each of the forward and reverse human growth hormone gene primers. PCR conditions were 95°C for 15 minutes, 32 cycles at 95°C for 30 sec, 55°C for 30 sec, and 72°C for 45 sec, with a final extension at 72°C for 10 minutes in a PTC – 200 (M.J Research, Inc; Watertown, Massachusettes, USA). The PCR product for each reaction was analysed by electrophoresis on 2% (w/v) agarose (SeaKem®GTG® Agarose, Lonza, Rockland, ME, USA) with ethidium bromide (AppliChem, Damstadt, Germany) staining and pictures taken using the Ultra-Violet Product (UVP) Biospectrum AC Imaging system with Biochemi-camera (AH Diagnostics, Cambridge, UK). The appearance of the 138 bp product in addition to 439 bp human growth hormone gene amplification fragment which served as positive internal control in each allele-specific reaction indicated the presence of that allele and a successful PCR (Appendix IIb).

#### 6.6.3.5 Genotyping of FcγR3B Variant

The human neutrophil antigen (HNA)-1a (*FCGR3B*1*), 1b (*FCGR3B*2*) and 1c (*FCGR3B*3*) variants formally known as NA1, NA2 and SH respectively were genotyped according to the protocol designed by (Tong *et al.,* 2003) with minor modifications. Each variant was genotyped using its own unique set of primer pair (DNA Technology, A/S, Denmark) in separate reactions as follows: HNA – 1a, sense (5'–CAGTGGTTTCACAATGTGAA-3') and antisense (5'-ATGGACTTCTAGCTGCAC-3'); HNA-1b, sense (5'–CAATGGTACAGCGTGCTT-3') and antisense (5'–TCTGTCGTTGACTGTGTCAG-3') and HNA-1c, sense (5'-AAGATCTCCCAAAGGCTGTG-3' and antisense (5'-TCTGTCGTTGACTGTGTCAT-3'). In addition, as was done in the FcγRIIIA PCR, the sense (5'–CAGTGCCTTCCCAACCATTCCCTTA-3') and antisense (5'-ATCCACTCACGGATTTCTGTTGTGTTTC-3') primers (DNA Technology, A/S, Denmark) specific for a 439bp fragment of human growth hormone were added to each reaction to serve as internal positive control and avoid false negatives. The 25µl reaction mixture for genotyping each allele contained, 40 ng genomic DNA, 2 units of TEMPase hotstart DNA polymerase with the corresponding 10X TEMpase buffer I (Ampliqon, BIOMOL GmbH, Hamburg, Germany), 2.5mM of each of the four dNTPs (Ampliqon, Germany), 20mM each of the FcγRIIIB – allele specific forward and reverse primer pair and 10mM each of the forward and reverse human growth hormone gene specific primer pair. The cycling conditions were 95°C activation for 15 minutes, 38 cycles of 94°C for 30 sec, 55°C for 30 sec, and 72°C

for 45 sec, with a final extension at 72°C for 7 minutes in a PTC – 200 (M.J Research, Inc; Watertown, Massachusettes, USA). The resulting PCR product for each reaction was analysed by electrophoresis on 2% (w/v) agarose (SeaKem®GTG® Agarose, Lonza, Rockland, ME, USA) with ethidium bromide (AppliChem, Damstadt, Germany) staining and pictures taken with the Ultra-Violet Product (UVP) Biospectrum AC Imaging system with Biochemi-camera (AH Diagnostics, Cambridge, UK). The appearance of a 141 bp, 156bp or 191bp PCR product in addition to 439 bp human growth hormone gene fragment for each allele-specific reaction indicated the presence the HNA-1a, HNA1b or HNA1c allele respectively and a successful PCR (Appendix IId).

#### 6.6.3.6 Genotyping of IgG3 Hinge Region Length Polymorphism

The IgG3 hinge region was amplified using the primers designed by Theisen *et al*., (unpublished). The sense 5' – (AAAACCCCACTTTGGTGACAC) and the antisense (5' - GGGTCCGGGAAATCATAAGG) primers (DNA Technology, A/S, Denmark), were 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 40ng genomic DNA, 10mM of each primer, 1.25mM of each dNTP, 1 unit of HotStarTaq® DNA polymerase and the corresponding 10X HotStar reaction buffer in a total volume of 25ul. The PCR conditions were 95°C activation for 15 minutes, 38 cycles of 95°C for 30 sec, 61°C for 30 sec, and 72°C for 30 s, with a final extension at 72°C for 7 min. The PCR

product was then analysed by electrophoresis on 2% (w/v) agarose (SeaKem®GTG® Agarose, Lonza, Rockland, ME, USA) with ethidium bromide (AppliChem, Damstadt, Germany) staining and pictures taken with the Ultra-Violet Product (UVP) Biospectrum AC Imaging system with Biochemi-camera (AH Diagnostics, Cambridge, UK). The appearance of 423, 611 and 807bp amplicons (Appendix IIe) correspond to short (S), medium (M) and long (L) hinge region phenotypes respectively.



### 6.7 Statistical Analysis

The clinical and epidemiological data were entered into a Microsoft Access based data management programme and upon alignment with genetics and immunological data, the final combined data set was analysed using STATA version 10.0 (Statcorp, Texas) and SigmaPlot version 11.0. Children were considered to have had a clinical malaria episode if they had a measured axillary temperature of > 37.5°C or a history of fever in the last 24 hours and parasitaemia of ≥ 2500/µl of blood. For each antigen, negative binomial regression was used to investigate the association between the levels of antibody measured at baseline and the incidence rate of clinical malaria. The levels of IgM, total IgG and IgG1-4 subclasses were analyzed for each antigen in turn. Age at enrolment was considered to be an important potential confounder and was included in the regressions as a factor. To increase specificity in defining individuals protected against and susceptible to clinical malaria, all individuals who reported sick and were treated for malaria with no

parasitaemia or parasite density below the threshold for the clinical malaria case definition used in this study were excluded from the final analysis. To construct a model with immunological variables independently associated with malaria risk, firstly, a model was produced for each antigen; in this, each antibody was a candidate for inclusion, provided that, when considered singly, the P value for association with malaria incidence was less than 0.15. Variables were then removed if the P value for the likelihood ratio test was more than 0.15 and provided that removal did not change the coefficients of variables in the model by more than 15%. Secondly, the variables in these models were combined in a final model in a similar way. Baseline parasitaemia was not considered as a potential confounder in the primary analyses. P value was considered statistically significant when $P \leq 0.05$. The distribution of the genotypes of the various polymorphisms and their associations with susceptibility or reduced risk to clinical malaria in the study populations was analysed by the Chi square test. P value considered statistically significant when $P \leq 0.05$, however, genotype data was only generated for all cohort 1 samples but not the entire samples of cohort 2 except those who were present in both cohort 1 and 2. Those polymorphisms which were found to be significantly associated with reduced risk from clinical malaria in the cohort studies were further analysed in the hospital-based case control study samples by considering all samples in cohort 1 as non-severe malaria controls. This allowed a testing of the contribution or otherwise of the polymorphisms in severe malaria pathogenesis. Proportions were compared by the chi square test.

# CHAPTER FOUR

## 6.0 RESULTS

### 6.1. Demographic and clinical characteristics of study populations

Data from 669 children ranging 1 to 12 years of age (cohort 1) and 550 individuals from 1 to 29 years of age (cohort 2) from 2 consecutive malaria transmission seasons were analysed in this study. The mean age of the two cohorts differed significantly (Table 4.1b) and there were approximately equal proportions of male and females in cohort 1 as there were in cohort 2. Blood group AB was the least observed 6.1% and 5.1% in cohorts 1 and 2 respectively while the proportions of the O blood group was the highest, 46.8% and 48.4 in cohorts 1 and 2 respectively. The incidence of clinical malaria (defined by parasitaemia >2500 and reported or measured axillary temperature >37.5ºC) was relatively low during the study periods for both cohorts, but was much lower in cohort 2 (4.80%) than cohort 1 (8.40%). The hemoglobin levels (g/dl) measured at baseline and that measured during acute malaria incidence within each cohort differed significantly (cohort 1: p=0.002; cohort 2: p=0.026; Mann-Whitney U test) (Table 4.1b).

In addition to the two cohort studies, data from a hospital based case-control study (n=182) in which patients were categorised into three malaria phenotypes: uncomplicated malaria (UM), severe malaria anaemia (SMA) and cerebral malaria were also analysed. The mean age and parasitaemia during acute malaria cases for individuals in the different disease categories of UM, SMA and CM was statistically

different ($p<0.001$ and $p=0.016$ respectively, Kruskal-Wallis One Way ANOVA) (Table 4.1a). The SMA group was the youngest [mean age = 3.0 (±0.4) years] while the oldest group was UM [mean age = 5.1 (±0.4) years], and the proportion of females in all the disease categories was higher than that of the males, being at least 52% in each.

**Table 4.1a: Characteristics of hospital-based case-control study population**

| **Characteristics** | **UM** | **SMA** | **CM** | **p-value** |
|---|---|---|---|---|
| No. of subjects (%) | 82 (45.1) | 41 (22.5) | 59 (32.4) | |
| Mean age (years) (± S.E) | 5.1 (± 0.4) | 3.0 (± 0.4) | 3.4 (± 0.3) | <0.001* |
| **Sex** | | | | |
| Male (%) | 39 (47.6) | 17 (41.5) | 27 (45.8) | |
| Female (%) | 43 (52.4) | 24 (58.5) | 32 (54.2) | |
| Parasitaemia at acute malaria, x $10^3$/ul (± S.E x $10^3$/ul) | 152.582 (± 53.201) | 68.382 (± 18.194) | 174.735 (± 34.905) | 0.016* |

** Kruskal-Wallis One Way ANOVA, UM: uncomplicated malaria; SMA: severe malaria anaemia; CM: cerebral malaria; S.E: standard error*

**Table 4.1b: Characteristics of cohort study populations**

| Characteristics | Cohort 1 (n=669) | Cohort 2 (n=550) | p-value |
|---|---|---|---|
| Mean age (years) (± S.E) | 5.5 (± 0.1) | 9.9 (± 0.3) | <0.001* |
| **Sex** | | | |
| Male (%) | 330 (49.3) | 269 (48.9) | |
| Female (%) | 339 (50.7) | 281 (51.1) | |
| **Blood Group** | | | |
| A (%) | 132 (19.7) | 100 (18.3) | |
| B (%) | 183 (27.4) | 165 (30.2) | |
| AB (%) | 41 (6.1) | 28 (5.1) | |
| O (%) | 313 (46.8) | 254 (46.4) | |
| O-to-A ratio | 2.37 | 2.54 | |
| *Malaria incidence rate (%)* | 8.40 | 4.80 | 0.170** |
| *Median Hb (g/dl) at baseline* | 11.6 | 12.3 | 0.002[!] |
| *Median Hb (g/dl) at acute malaria* | 11.1 | 11.9 | 0.029[!!] |
| **Bednet use** | | | |
| Yes (%) | 273 (40.8) | 122 (22.3) | |
| No (%) | 396 (59.2) | 426 (77.7) | |
| **Sickle Status** | | | |
| Negative (%) | 569 (85.1) | 472 (89.1) | |
| Positive (%) | 99 (14.8) | 58 (10.9) | |

**Mann-Whitney U test for age differences between the two cohorts, Mann-Whitney U test between Hb at baseline and Hb at acute malaria incidence for cohort 1-! and cohort 2-!! respectively; ** one-sample t-test; S.E, standard error*

### 6.2. Antigen-specific antibody levels

The reference standard curves used in transforming all optical density (OD) values in the ELISA to antibody units were optimized for each antigen such that the same antibody unit of 5 was assigned to an OD of 0.5 in all antibody measurements irrespective of the antigen being tested thus, allowing for a comparison of antibody levels to different antigens on a uniform scale. The level of antigen-specific antibody response was compared on the bases of IgG and IgM responses (Figure 4.1) for all the antigens. IgG and IgM levels for the various antigens differed significantly ($p \leq 0.001$) and levels of these antibodies to AMA1-FVO were the highest (Figure 4.1). IgG and IgM levels to the peptide antigens AS202.11 and LR146 respectively, were the lowest. The IgG levels of the antigens in decreasing order was; AMA1-FVO, EBA-175, MSP3-FVO, GLURP R2, GLURP R0, MSP1-hybrid, LR146 and AS202.11, respectively. On the other hand, the trend for IgM levels of the antigens in decreasing order was; AMA1-FVO, GLURP R0, EBA-175, AS202.11, GLURP R2, MSP3-FVO, MSP1-hybrid and LR146. For each antigen, there was a statistically significant difference between IgG responses and that of IgM ($p < 0.001$, Mann-Whitney U Test) except for EBA 175 for which the isotype levels were not significantly different ($p = 0.535$, Mann-Whitney U Test).

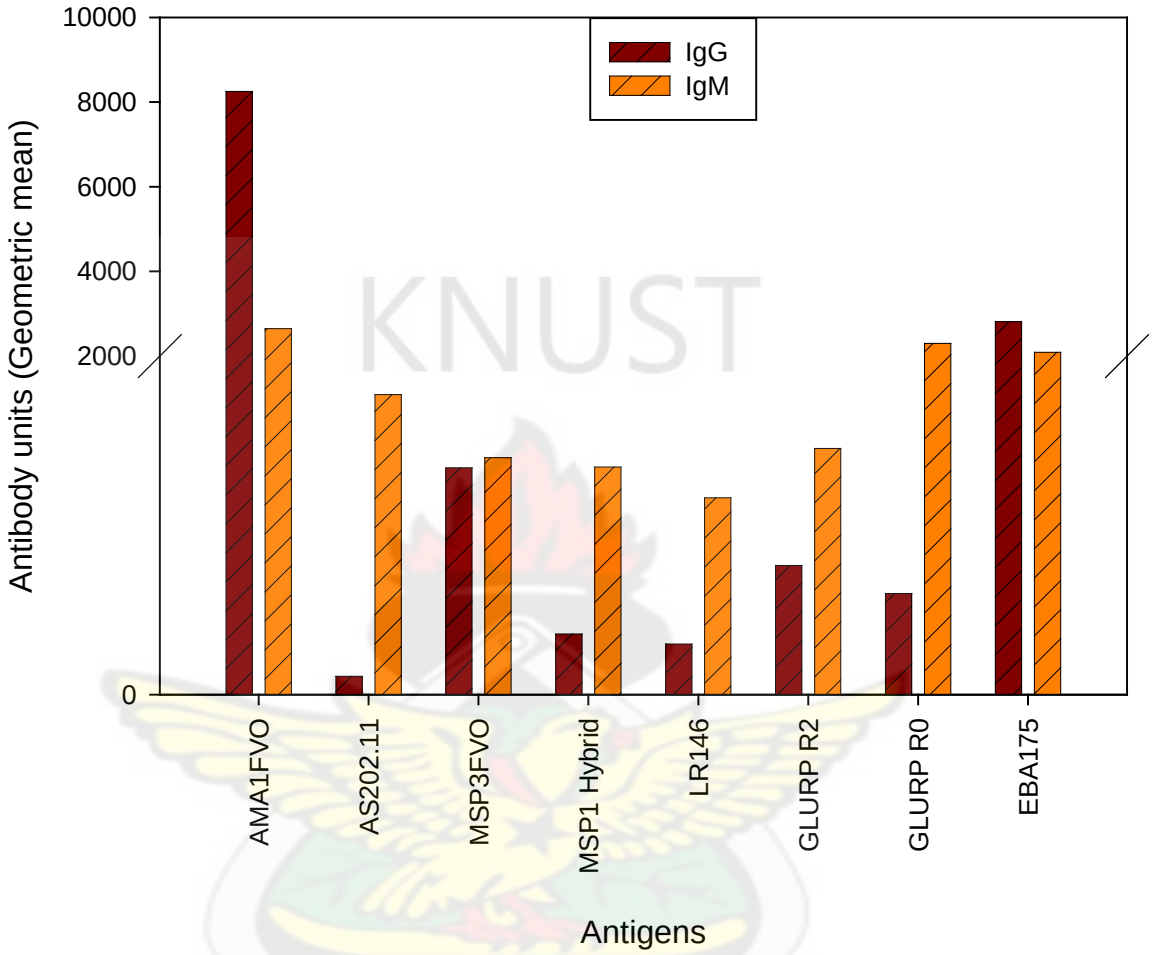



**Figure 4.1 Antigen specific isotype IgG and IgM responses**

### 6.3. Pattern of IgG1-4 subclass immune responses

The pattern of IgG1-4 subclass immune responses was assessed for each of the recombinant antigen but not for the peptide antigens (LR146 and AS202.11). This is because the peptides were found to elicit predominantly, IgM and relatively low IgG responses as shown in Figure 4.1. The most predominant IgG subclass antibody was IgG1 which was the highest for all the antigens except MSP1 hybrid and GLURP-R0 (Figure 4.2) for which IgG2 responses were higher than the other IgG subclasses. The major subclass response for MSP1 hybrid was IgG2, however, when compared with IgG2 levels for the other antigens tested it was the lowest. The highest level of IgG1 response was found in MSP3-FVO followed by EBA-175, AMA1-FVO and GLURP-R2 respectively. IgG3 response was also found to be the highest in MSP3-FVO but the lowest in MSP1 hybrid which levels were similar to that in AMA1-FVO. Levels of IgG4 antibodies was the lowest recorded for all the antigens with the least found in GLURP-R0 (Figure 4.2). Of all the antigens, MSP3-FVO showed the highest IgG1-4 subclass response while the least was recorded in MSP1-hybrid. The levels of IgG1-4 subclass antibodies for each antigen was significantly different ($p < 0.001$, Kruskal-Wallis One Way ANOVA) and this was also found to be true ($p < 0.05$) when a pairwise multiple comparison test (Tukey test) was applied in an attempt to identify antibody pairs whose levels did not differ for any particular antigen.

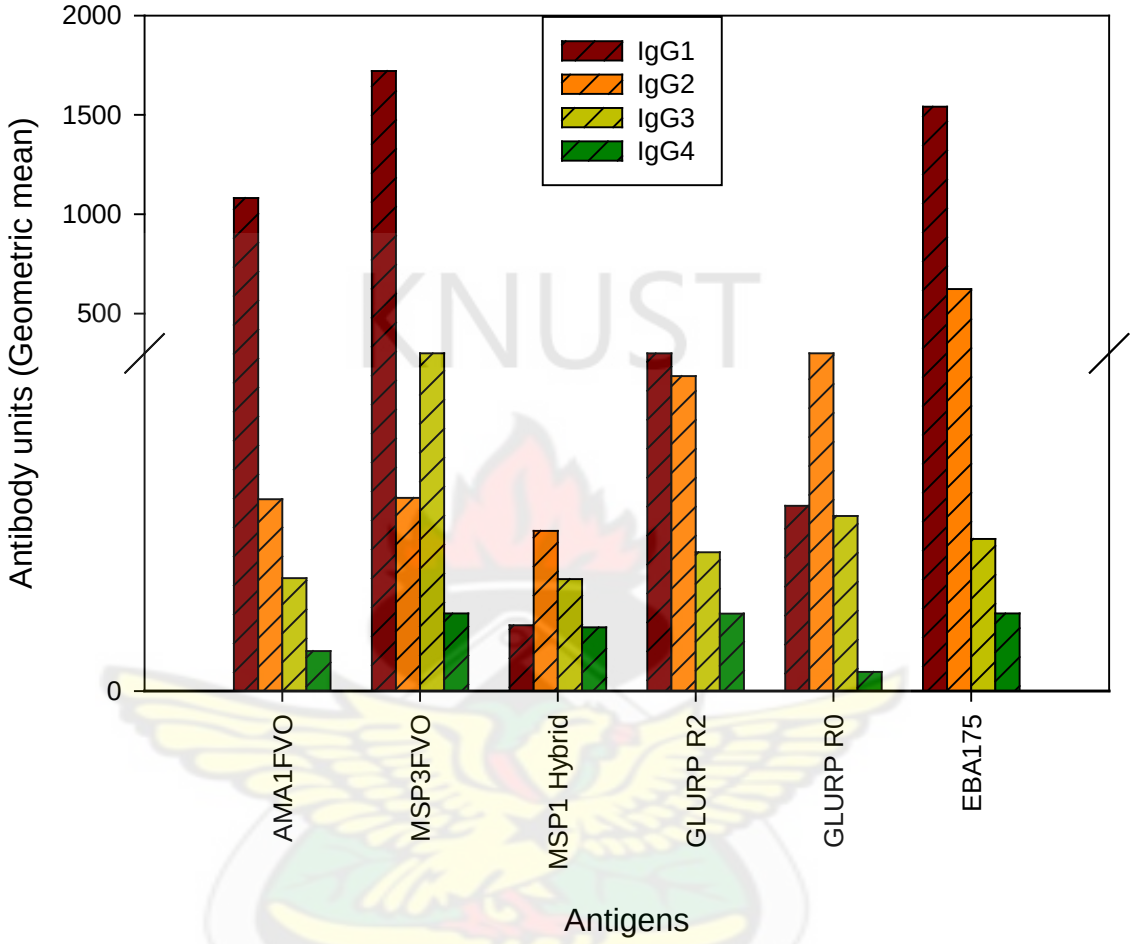



**Figure 4.2 Antigen specific IgG1-4 subclass responses**

### 6.4. Relationship between malaria antigen-specific antibody responses with age

The study found a general trend of increase in IgG levels with age to all the antigens (Figure 4.3a). However, the correlation with age for IgG levels was weak for AMA1 FVO, LR146, GLURP R2 and MSP1 hybrid ($0.187 \leq r \leq 0.092$, $p<0.032$) but moderate for MSP3 FVO ($r = 0.298$, $p< 0.00001$) and EBA-175 ($r = 0.387$, $p <0.00001$). On the other hand, IgM levels for GLURP R2 and EBA-175 were weakly correlated with age of the participants screened ($r = 0.163$, $p=0.0001$ and $r = 0.154$, $p = 0.0003$ respectively) while levels of IgM for the other recombinant antigens, MSP3 FVO, MSP1 hybrid, GLURP-R2, GLURP-R0, AMA1-FVO and EBA-175 moderately correlated with age ($0.213 \leq r \leq 0.260$, $p<0.00001$). IgM antibodies to the peptide antigens LR146 ($r=0.057$, $p=0.184$) and AS202.11 ($r=0.077$, $p=0.071$) were not found to significantly correlate with age of study participants. There was statistically significant difference ($p < 0.0001$; Mann-Whitney U Test) between levels of IgG and IgM for each antigen except for EBA-175 ($p = 0.535$; Mann-Whitney U Test) which did not show significant difference between levels of the two antibodies types. To further explore the pattern of antigen specific IgG and IgM responses in different age groups, the subjects were categorized into four groups according to age as follows: 1 to 5 years, n=132; 6 to 10 years, n=139; 11 to 15 years, n=98 and 16 to 29 years, n=92 respectively. For each of the antigens, there was a consistent trend of increasing levels of both IgG and IgM with age groups (Figure 4.3a and 4.3b) and the difference was found to be statistically significant ($p \leq 0.001$, Kruskal-Wallis One Way ANOVA). In order to

identify the age groups that differ in antibody levels for each antigen, a pair-wise multiple comparison test (Dunn's method) was used. IgM levels for the 16 to 29 years group was always lower than the 11 to 15 years group (Figure 4.3b) but this difference was only statistically significant ($p > 0.05$) for GLURP-R2 and EBA-175 ($p < 0.05$). There was no difference, ($p > 0.05$) in IgG levels to the antigens AS202.11, LR146 and MSP1 hybrid between the age groups; 6 to 10 years and 11 to 15 year. IgG levels to AMA1-FVO and EBA-175 between the age groups (11 to 15 years) and (16 to 29 years) were also not significantly different.

Furthermore, IgG1-4 subclass levels for most of the antigens showed a statistically significant positive correlation with age. IgG2, 3 and 4 for AMA1-FVO, IgG1 and 4 for GLURP-R2; IgG1, 2 and 4 for GLURP-R0; IgG4 and 2 for MSP3-FVO; IgG3 and 4 for EBA-175 and IgG2 and 3 for MSP1 hybrid were weakly correlated with age ($0.197 \leq r \leq 0.090$, $p<0.036$). There was, however, a strong correlation between IgG1 and 3 for MSP3-FVO; IgG2 and 3 for GLURP-R2; IgG1 and 2 for AMA1-FVO and IgG1 and 2 for EBA-175 with age ($0.413 \leq r \leq 0.202$, $p < 0.00001$).

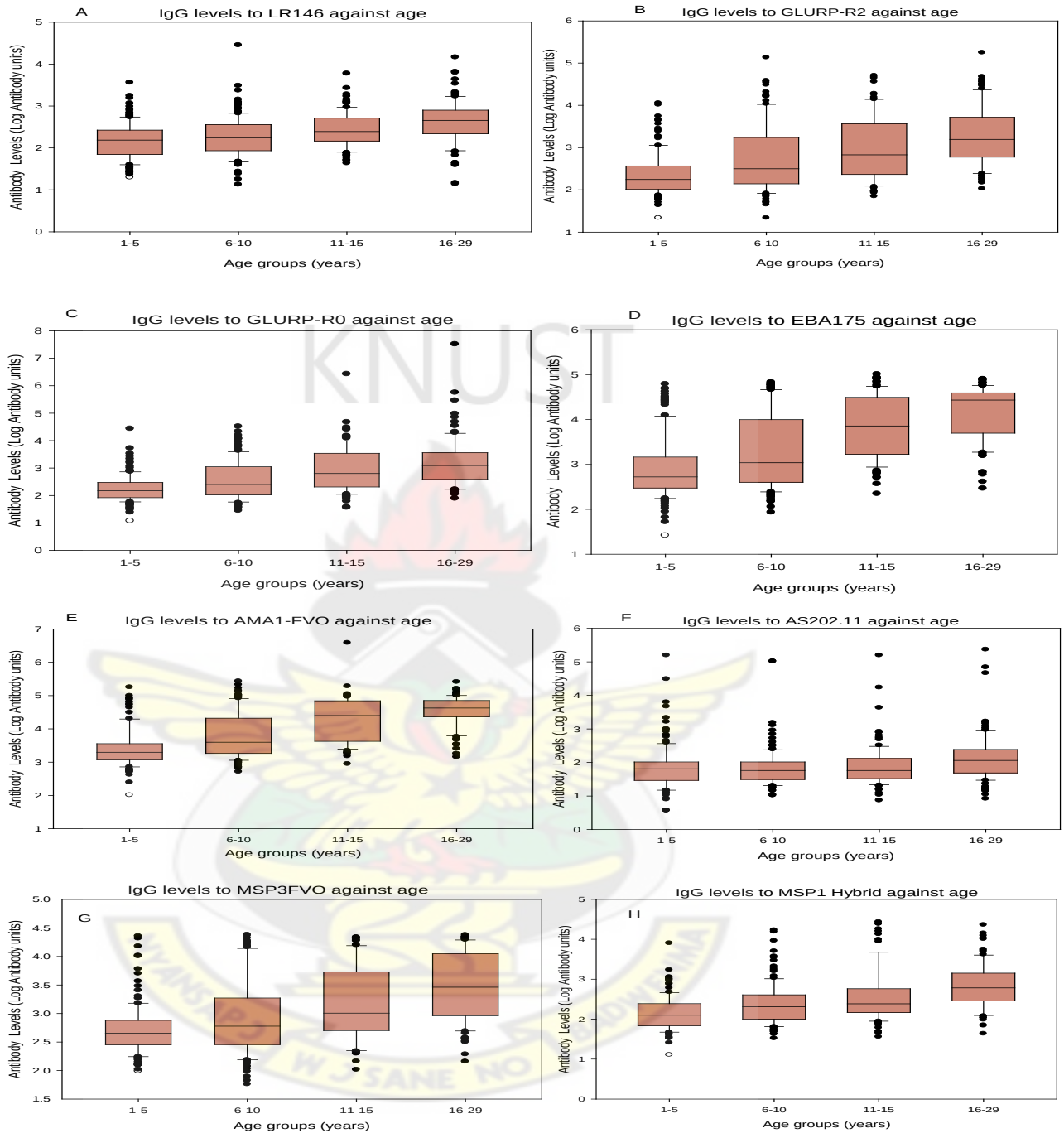



**Figure 4.3a Antigen specific IgG responses with age.**

*IgG responses to each of the antigens LR146, GLURP-R2, GLURP-R0, EBA-175, AMA1-FVO, AS202.11, MSP3-FV0 and MSP1-hybrid are shown separately in plots A; B; C; D; E; F; G and H respectively. IgG antibody units were log transformed and plotted against the age groupings (1-5; 6-10; 11-15; 16-29) years of individuals.*

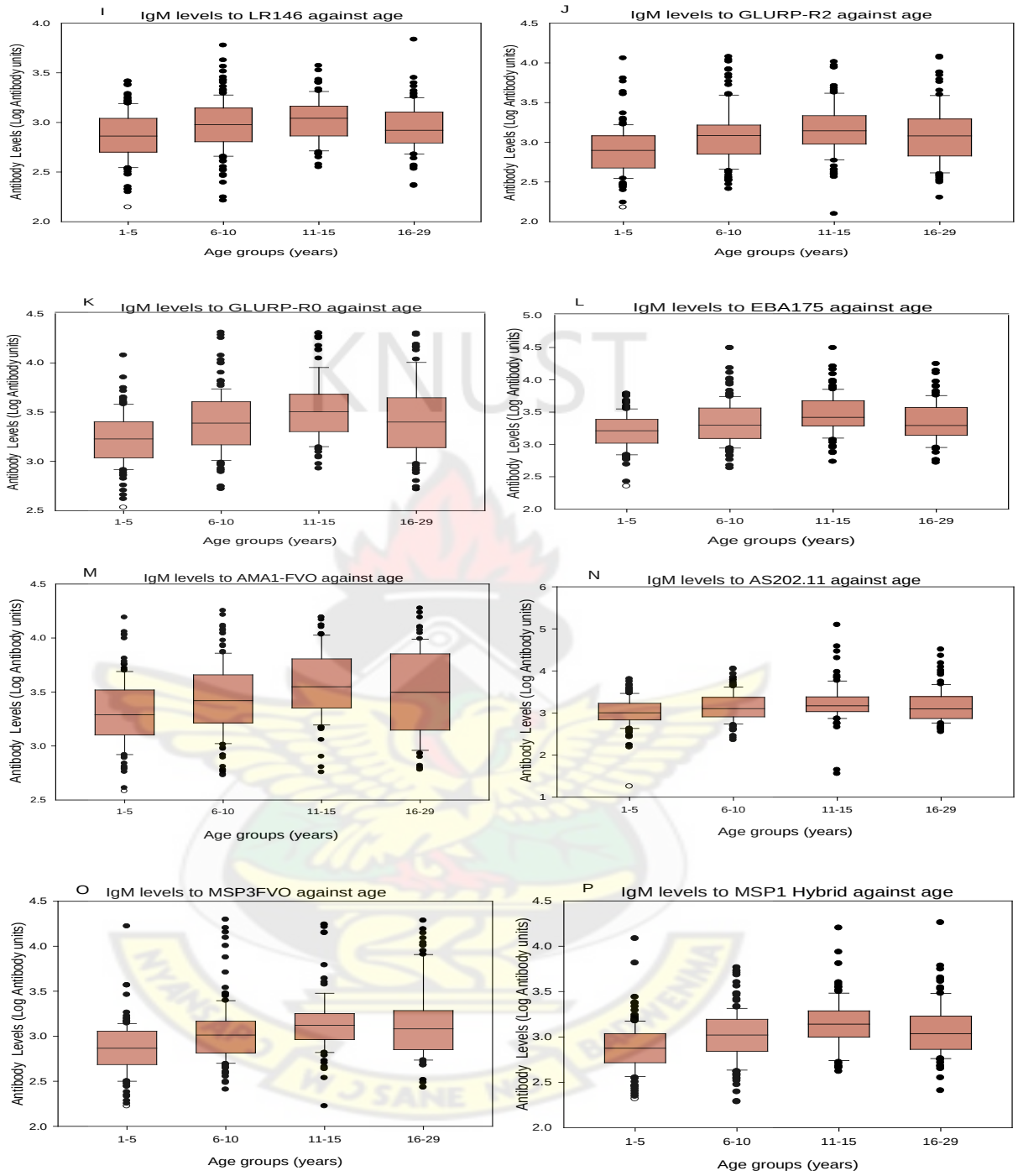



**Figure 4.3b Antigen specific IgM responses with age.**

*IgM responses to each of the antigens LR146, GLURP-R2, GLURP-R0, EBA-175, AMA1-FVO, AS202.11, MSP3-FV0 and MSP1-hybrid are shown separately in plots I; J; K; L; M; N; O and P respectively. IgM antibody units were log transformed and plotted against the age groupings (1-5; 6-10; 11-15; 16-29) years of individuals.*

### 6.5. Age, acute case parasitaemia and hemoglobin levels

Data from both cohort 1 and 2, suggest that older individuals tend to have lower parasite load during acute malaria episodes compared to younger ones (Figure 4.4). On the other hand Hb levels measured at acute malaria episodes increased with age of participants in both cohorts but was only significant in cohort 1. A comparison between parasite load and Hb levels during acute malaria episodes did not show any significant associations. The general trend observed in both studies (cohort 1 and 2) was that acute case parasitaemia decreased with age while Hb levels in acute malaria episodes seemed to increase with age (Figure 4.4). To further examine the effect of age on parasite densities and Hb levels during acute malaria cases, a hospital-based case control study was considered where patients were categorized into severe/complicated malaria (n=120) and cerebral malaria (n=58). A statistically significant positive correlation ($r=0.443$, $p<0.00001$) was observed between age and Hb levels in both patient groups.

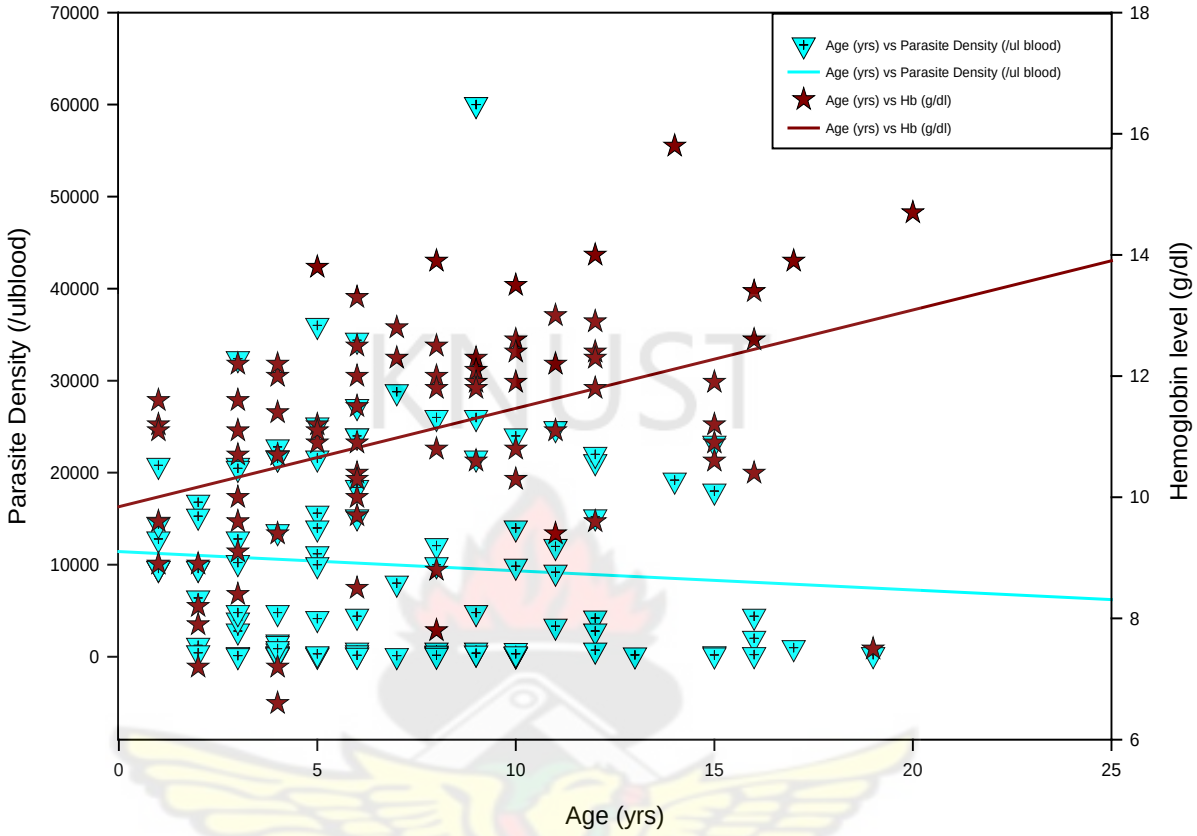



**Figure 4.4 Distribution of acute case parasitaemia and hemoglobin with age in cohorts 1 and 2 combined**

### 6.6. Association between antibody levels and clinical malaria

In this study, malaria was defined as history of fever or temperature >37.5°C and parasite density ≥ 2500/µl though other parasitaemia thresholds such as ≥ 5000/µl have been used in other studies (Smith *et al.,* 1994; Okenu *et al.,* 2000; Mwangi *et al.,* 2005). The parasitaemia cut-off for this study was chosen to be at least ten times greater than the geometric mean asymptomatic parasitaemia determined prior to the transmission season (baseline parasitaemia). Of all the eight antigens tested for IgG, IgM and IgG1 to 4 subclasses, higher baseline antibody levels were recorded for individuals who did not fall sick than for those who reported sick during the period of the study. The differences were not statistically significant at the parasitaemia for malaria case definition used when the confounding effect of age was adjusted for in a univariate analysis. However, IgG1 to MSP3 FVO (IRR=0.83 [95% CI, 0.64, 1.06, p= 0.13]), IgG3 to GLURP R0 (IRR= 0.89 [95% CI, 0.77, 1.03, p=0.12], IgG3 to EBA-175 (IRR=0.88 [95% CI, 0.73, 1.05, p= 0.14]) and IgG4 to MSP3 FVO (IRR=0.81 [95% CI, 0.65, 1.02, p= 0.08]) (Table 4.2) showed trends towards association with protection against clinical malaria. In a final analysis using a model that comprises, age and the immunological variables with trends suggestive of protection, only IgG4 to MSP3 FVO (IRR=0.85 [95% CI, 0.66, 1.08, p= 0.19]) and IgG3 to GLURP R0 (IRR= 0.92 [95% CI, 0.79, 1.07, p=0.29] showed persistent trends towards association with protection though statistically not significant (Table 4.3).

**Table 4.2: Age-adjusted incidence rate ratios for the association between antibody concentration and clinical malaria**

| Antibody | Antigen | Crude IRR (95%CI) | IRR adjusted for age (95%CI) | P-value for adjusted IRR |
|---|---|---|---|---|
| IgG | GLURP R0 | 0.89 (0.73, 1.09) | 0.89 (0.72, 1.10) | 0.29 |
| | GLURP R2 | 0.90 (0.74, 1.10) | 0.91 (0.74, 1.11) | 0.35 |
| | MSP3 FVO | 0.83 (0.66, 1.05) | 0.85 (0.67, 1.08) | 0.18 |
| | MSP1 hybrid | 0.85 (0.65, 1.11) | 0.86 (0.65, 1.14) | 0.29 |
| | AS202.11 | 1.03 (0.84, 1.26) | 1.06 (0.87, 1.30) | 0.54 |
| | AMA1 FVO | 0.99 (0.83, 1.19) | 1.00 (0.82, 1.22) | 0.97 |
| | EBA-175 | 1.06 (0.91, 1.23) | 1.09 (0.92, 1.30) | 0.31 |
| | LR146 | 1.00 (0.76, 1.22) | 1.05 (0.78, 1.41) | 0.73 |
| IgG1 | GLURP R0 | 0.89 (0.72, 1.10) | 0.88 (0.70, 1.09) | 0.24 |
| | GLURP R2 | 0.99 (0.80, 1.21) | 0.99 (0.80, 1.21) | 0.89 |
| | **MSP3 FVO** | **0.80 (0.62, 1.03)** | **0.83 (0.64, 1.06)** | **0.13** |
| | MSP1 hybrid | 0.95 (0.81, 1.11) | 0.96 (0.81, 1.12) | 0.59 |
| | AMA1 FVO | 1.01 (0.89, 1.16) | 1.03 (0.89, 1.19) | 0.67 |
| | EBA-175 | 0.97 (0.83, 1.13) | 0.98 (0.83, 1.16) | 0.85 |
| IgG2 | GLURP R0 | 0.93 (0.75, 1.15) | 0.96 (0.77, 1.20) | 0.71 |
| | GLURP R2 | 0.92 (0.74, 1.15) | 0.93 (0.73, 1.18) | 0.56 |
| | MSP3 FVO | 0.90 (0.72, 1.12) | 0.90 (0.72, 1.13) | 0.37 |
| | MSP1 hybrid | 0.89 (0.67, 1.18) | 0.93 (0.70, 1.24) | 0.63 |
| | AMA1 FVO | 0.93 (0.74, 1.16) | 0.93 (0.73, 1.19) | 0.58 |
| | EBA-175 | 0.89 (0.63, 1.25) | 0.89 (0.61, 1.31) | 0.56 |
| IgG3 | **GLURP R0** | **0.90 (0.78, 1.03)** | **0.89 (0.77, 1.03)** | **0.12** |
| | GLURP R2 | 0.93 (0.80, 1.07) | 0.93 (0.80, 1.09) | 0.38 |
| | MSP3 FVO | 0.89 (0.76, 1.05) | 0.90 (0.76, 1.07) | 0.22 |
| | MSP1 hybrid | 0.85 (0.67, 1.07) | 0.86 (0.66, 1.09) | 0.2 |
| | AMA1 FVO | 0.93 (0.78, 1.09) | 0.93 (0.78, 1.10) | 0.41 |
| | **EBA 175** | **0.87 (0.73, 1.03)** | **0.88 (0.73, 1.05)** | **0.14** |
| IgG4 | GLURP R0 | 0.94 (0.77, 1.16) | 0.94 (0.74, 1.19) | 0.61 |
| | GLURP R2 | 0.81 (0.57, 1.14) | 0.81 (0.56, 1.16) | 0.25 |
| | **MSP3 FVO** | **0.83 (0.66, 1.03)** | **0.81 (0.65, 1.02)** | **0.08** |
| | MSP1 hybrid | 0.83 (0.57, 1.22) | 0.83 (0.57, 1.22) | 0.35 |
| | AMA1 FVO | 1.04 (0.91, 1.21) | 1.05 (0.90, 1.23) | 0.52 |
| | EBA-175 | 0.91 (0.76, 1.09) | 0.93 (0.76, 1.11) | 0.39 |
| IgM | GLURP R0 | 1.09 (0.74, 1.61) | 1.03 (0.69, 1.55) | 0.88 |
| | GLURP R2 | 1.00 (0.68, 1.48) | 0.94 (0.61, 1.40) | 0.72 |
| | MSP3 FVO | 0.85 (0.56, 1.28) | 0.76 (0.48, 1.22) | 0.26 |
| | MSP1 hybrid | 1.18 (0.78, 1.78) | 1.15 (0.74, 1.79) | 0.54 |
| | AS202.11 | 1.07 (0.76, 1.52) | 1.02 (0.72, 1.46) | 0.89 |
| | AMA1 FVO | 0.99 (0.68, 1.43) | 0.93 (0.62, 1.38) | 0.71 |
| | EBA-175 | 0.94 (0.65, 1.37) | 0.87 (0.60, 1.28) | 0.49 |
| | LR146 | 1.12 (0.69, 1.84) | 1.03 (0.63, 1.69) | 0.89 |

*IRRs indicate the ratio of malaria incidence rates associated with a twofold increase in antibody level.Malaria case is defined as history of fever or temperature>37.5 and parasite density>=2500u/l*

**Table 4.3: Adjusted incidence rate ratios for immunological variables independently associated with malaria risk in the final model**

| Immunological variables | | Adjusted IRR (95%CI) | Wald P-value | LR test |
|---|---|---|---|---|
| MSP3 IgG4 | Values transformed to log base 2 | 0.85 (0.66, 1.08) | P=0.19 | |
| GLURP R0 IgG3 | Values transformed to log base 2 | 0.92 (0.79, 1.07) | P=0.29 | |
| Age group | 1-5yrs | 1 | | $\chi^2$=7.40, P=0.06 |
| | 6-10yrs | 2.14 (0.72, 6.33) | P=0.17 | |
| | 10-15yrs | 3.54 (1.10, 11.41) | P=0.03 | |
| | 16-29yrs | 0.51 (0.06, 4.77) | P=0.56 | |

*IRR indicates the ratio of malaria incidence rates associated with a twofold increase in antibody level Parasite cut off is 2500+. MSP3 IgG4 means IgG4 subclass specific antibody to the merozoite surface protein 3; GLURP R0 IgG3 means IgG3 subclass specific antibody to the glutamate-rich protein. The incidence rate was calculated as the total number of episodes divided by the total person year at risk.*

## 6.7. FcγRIIA, FcγRIIB, FcγRIIIA and FcγRIIIB genotype distribution in Ghanaian children

The genes for FcγRIIA, FcγRIIB, FcγRIIIA and FcγRIIIB from 669 Ghanaian children were screened for polymorphisms using PCR and sequencing based methods (Table 4.4). The frequencies of the FcγRIIA and FcγRIIB genotypes were found to be significantly different from those predicted from the Hardy-Weinberg equilibrium, using $\chi^2$ analysis (Table 4.4). The observed frequency of FcγRIIA heterozygotes exceeded the expected frequency, whereas the observed frequency of FcγRIIB heterozygotes was lower than expected (Table 4.4). Since previous studies (Hatta *et al.*, 1999; Kyogoku *et al.*, 2002) have described linkage disequilibrium between the FcγIIA-131 H/R and FcγIIIA-158 V/F alleles, the distribution of allelic variations in FcγRIIA, FcγRIIA, FcγRIIIA was calculated (Table 4.5). There was strong evidence for a non-random distribution of combinations in FcγRIIA and FcγRIIIA ($P < 0.001$, $\chi^2$ analysis), FcγRIIA and FcγRIIA ($P = 0.0024$, $\chi^2$ analysis), and FcγRIIB and FcγRIIIA ($P < 0.001$, $\chi^2$ analysis) suggesting an overrepresentation of one or more combinations of variant genotypes. As expected, the estimated haplotype frequencies of VH and RF exceeded the expected frequencies (Table 4.6). Moreover, the VH haplotype was found to be linked to FcγRIIB-232 I, whereas the RF haplotype seemed to be preferentially linked to FcγRIIB-232 T (Table 4.6).

### 6.8. FcγR genotype and clinical malaria

In order to determine whether any of the FcγR genotypes were associated with clinical malaria, the genotypes were calculated in children with and without clinical malaria (Tabel 4.7). In contrast to previous findings, this study did not find a significant skewing in the FcγRIIA genotype frequency towards protection or susceptibility to clinical malaria in Ghanaian children. The only significant difference was observed in FcγRIIIB. The genotype 1/1 was overrepresented in protected children whereas the 2/2 genotype was overrepresented in children who have experienced at least an episode of clinical malaria (P=0.007, $\chi^2$ analysis). Interestingly, of the 95 children who were typed as $1^-/2^-$; ninety-two (15.1 %) were in the group of protected children whereas only three (5.4%) children had experienced one or more episodes of clinical malaria. These three children were all typed as $3^-$ suggesting that they lack the FcγRIIIB gene. Of the remaining $1^-/2^-$ children, twenty-three (3.5%) were also $3^-$ suggesting that this allele has no impact on clinical malaria.

### 6.9. FcγRIIIB genotypes in severe malaria

In order to examine whether FcγRIIIB genotype frequencies may also be observed in children with more complicated forms of malaria, the gene for FcγRIIIB was typed in 178 children and the study found a skewing of FcγRIIIB genotype frequencies in children who have been hospitalized with complicated malaria. From

Table 4.8, there is a significant skewing in the FcγRIIIB genotype frequency in children with complicated malaria as compared to the controls (P=0.0002, $\chi^2$ analysis). Children with severe anaemia and or cerebral malaria showed an underrepresentation of the 1/1 genotype and an overrepresentation of the 2/2 genotype. Thus, confirming the "protective" effect of the FcγRIIIB 1/1 genotype.

**Table 4.4. Distribution of FcγIIA (A), FcγIIB (B), FcγIIIA (C) and FcγIIIB (D) genotypes in Ghanaian children.**

A

| **FcγRIIA-131H/R genotype frequency n (%)** | | | | **p-value** | **Allele frequency** | |
|---|---|---|---|---|---|---|
| | RR | RH | HH | | R | H |
| All | 200 (30.0) | 360 (54.0) | 107 (16.0) | | 0.57 | 0.43 |
| Chi square | | | | < 0.001 | | |

B

| **FcγRIIB-232I/T genotype frequency n (%)** | | | | **p-value** | **Allele frequency** | |
|---|---|---|---|---|---|---|
| | II | IT | TT | | I | T |
| All | 390 (59.5) | 190 (29.0) | 75 (11.5) | | 0.74 | 0.26 |
| Chi square | | | | < 0.001 | | |

C

| **FcγRIIIA-158F/V genotype frequency n (%)** | | | | **p-value** | **Allele frequency** | |
|---|---|---|---|---|---|---|
| | FF | FV | VV | | F | V |
| All | 339 (51.1) | 268 (40.4) | 56 (8.4) | | 0.71 | 0.29 |
| Chi square | | | | > 0.05 | | |

D

| **FcγRIIIB-NA1/NA2 genotype frequency n (%)** | | | | | **p-value** | **Allele frequency** | |
|---|---|---|---|---|---|---|---|
| | 1/1 | 1/2 | 2/2 | 1-/2- | | 1 | 2 |
| All | 240 (36.1) | 164 (24.5) | 168 (25.1) | 95 (14.3) | | 0.71 | 0.29 |
| Chi square | | | | | <0.001 | | |

*R-Arginine; H-Histidine; I-isoleucine; T-Threonine; F- Phenylalanine; V-Valine, 1-Neutrophil Antigen (NA1); 2- Neutrophil Antigen (NA2)*

**Table 4.5. Linkage Disequilibrium between FcγIIA-131 H/R, FcγIIIA-158 V/F, and FcγIIB I/T alleles.**

| | FcγRIIA vrs FcγRIIIA | | | | FcγRIIA vrs FcγRIIB | | | | FcγRIIB vrs FcγRIIIA | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | no | % | p value | | no | % | p value | | no | % | p value |
| No. | 663 | | | No. | 655 | | | No. | 653 | | |
| HH | | | | HH | | | | II | | | |
| VV | 25 | 3.8 | | II | 76 | 11.6 | | VV | 45 | 6.9 | |
| VF | 58 | 8.7 | | IT | 26 | 4 | | VF | 169 | 25.9 | |
| FF | 23 | 3.5 | | TT | 4 | 0.6 | | FF | 174 | 26.6 | |
| HR | | | | HR | | | | IT | | | |
| VV | 30 | 4.5 | | II | 210 | 32.1 | | VV | 8 | 1.2 | |
| VF | 163 | 24.6 | | IT | 105 | 16 | | VF | 73 | 11.2 | |
| FF | 164 | 24.7 | | TT | 37 | 5.6 | | FF | 109 | 16.7 | |
| RR | | | | RR | | | | TT | | | |
| VV | 1 | 0.2 | | II | 104 | 15.9 | | VV | 2 | 0.3 | |
| VF | 47 | 7.1 | | IT | 59 | 9 | | VF | 22 | 3.4 | |
| FF | 152 | 22.9 | | TT | 34 | 5.2 | | FF | 51 | 7.8 | |
| Chi square | | | <0.001 | | | | 0.0024 | | | | <0.001 |

*R-Arginine; H-Histidine; I-isoleucine; T-Threonine; F- Phenylalanine; V-Valine*

**Table 4.6. Haplotype frequencies.**

| Haplotype | Estimated frequency | Expected frequency |
|---|---|---|
| H | | |
| V | **0.210** | 0.123 |
| F | 0.219 | 0.307 |
| R | | |
| V | 0.077 | 0.164 |
| F | **0.494** | 0.406 |
| HV | | |
| I | **0.1847** | 0.0913 |
| T | 0.0269 | 0.0321 |
| HF | | |
| I | 0.1631 | 0.2269 |
| T | 0.0562 | 0.0797 |
| RV | | |
| I | 0.0609 | 0.1211 |
| T | 0.0148 | 0.0425 |
| RF | | |
| I | **0.3309** | 0.3007 |
| T | **0.1625** | 0.1057 |

*R-Arginine; H-Histidine; I-isoleucine; T-Threonine; F- Phenylalanine; V-Valine*

**Table 4.7. Distribution of FcγIIA (A), FcγIIB (B), FcγIIIA (C) and FcγIIIB (D) genotypes in children susceptible to and protected from clinical malaria.**

| Genotype | Susceptible | | Protected | | p-value |
|---|---|---|---|---|---|
| **A** | No. | % | No. | % | |
| FcγRIIA-131H/R | | | | | |
| RR | 18 | 31.6 | 182 | 29.8 | |
| RH | 33 | 57.9 | 327 | 53.6 | |
| HH | 6 | 10.5 | 101 | 16.6 | |
| **B** | | | | | 0.494 |
| FcγRIIB-232I/T | | | | | |
| II | 30 | 53.6 | 360 | 60.1 | |
| IT | 17 | 30.4 | 173 | 28.9 | |
| TT | 9 | 16.1 | 66 | 11.0 | |
| **C** | | | | | 0.461 |
| FcγRIIIA158F/V | | | | | |
| FF | 32 | 57.1 | 307 | 50.6 | |
| FV | 16 | 28.6 | 252 | 41.5 | |
| VV | 8 | 14.3 | 48 | 7.9 | |
| **D** | | | | | 0.081 |
| FcγRIIIB-NA1/NA2 | | | | | |
| 1/1 | 16 | 28.6 | 223 | 36.6 | |
| 1/2 | 13 | 23.2 | 151 | 24.8 | |
| 2/2 | 24 | 42.9 | 143 | 23.5 | |
| $1^{-}/2^{-}$ | 3 | 5.4 | 92 | 15.1 | |
| | | | | | 0.007 |

*R-Arginine; H-Histidine; I-isoleucine; T-Threonine; F- Phenylalanine; V-Valine, 1-Neutrophil Antigen (NA1); 2- Neutrophil Antigen (NA2)*

**Table 4.8 Distribution of FcγIIIB-NA1/NA2 genotypes in Ghanaian children with severe malaria and controls**

| Genotype | Controls [a] n=665 | Hospital n=178 | Severe malaria anaemia n=120 | Cerebral malaria n=58 | p-value |
|---|---|---|---|---|---|
| $2^-/1^-$ | 95 (14.3) | 39 (21.9) | 30 (25.0) | 9 (15.5) | |
| 1/1 | 240 (36.1) | 48 (27.0) | 31 (25.8) | 17 (29.3) | |
| 2/2 | 167 (25.1) | 59 (33.1) | 37 (30.8) | 22 (37.9) | |
| 1/2 | 163 (24.5) | 32 (18.0) | 22 (18.3) | 10 (17.2) | |
| Chi square | | | | | 0.0002 |

[a] *All children from the longitudinal community-based study – cohort 1; 1-Neutrophil Antigen (NA1); 2- Neutrophil Antigen (NA2)*

## 6.10. IgG3 hinge region length polymorphism distribution in Ghanaian children and association with clinical malaria

Length polymorphism of the IgG3 hinge region was genotyped for a 252 sub-population of study participants who were present in both cohorts using the PCR technique. The most predominant allele was the long (L) which was present in 63% of the individuals genotyped while the heterozygous (LM) was the genotype with the highest frequency (46.03%) and the homozygous medium (MM) was the least being present in 13.89% of the samples. However, the observed genotypic frequencies were not statistically different from those predicted by the Hardy-Weinberg equilibrium ($p > 0.05$, $\chi^2$ analysis) (Table 4.9).

**Table 4.9 Distribution of IgG3 hinge region length polymorphism in Ghanaian children**

| | Genotype frequency n (%) | | | p-value | Allele frequency | |
|---|---|---|---|---|---|---|
| | LL | LM | MM | | L | M |
| All | 101(40.08) | 116(46.03) | 35(13.89) | | 0.63 | 0.37 |
| HW Chi square | | | | > 0.05 | | |

*L-long hinge region allele, M-medium hinge region allele*

The genotype distribution was also assessed in individuals apparently protected against and those susceptible to clinical malaria. There was no significant difference ($p = 0.67$, $\chi^2$ analysis) (Table 4.10) in the genotype distribution between those susceptible to and those protected against clinical malaria. However, a greater proportion of the susceptible group had the LL genotype while the MM genotype was the least recorded (11.1%) among the same group.

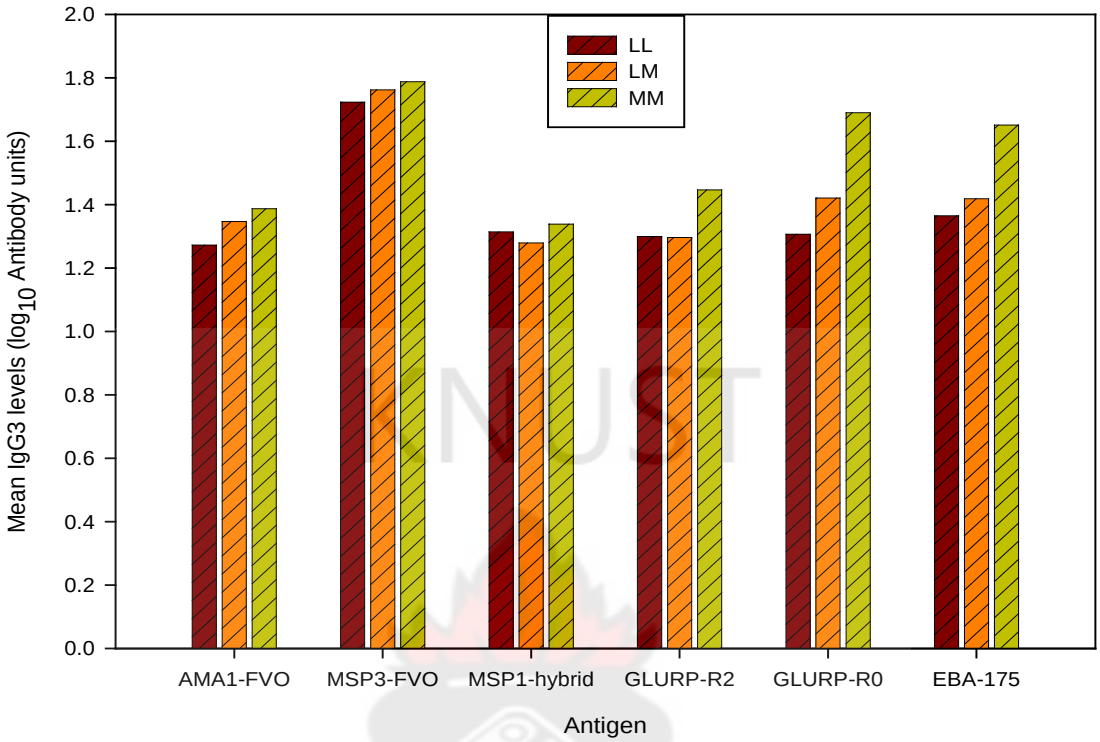



**Figure 4.5 Antigen specific IgG3 levels and IgG3 hinge region length polymorphism**

**Table 4.10 Distribution of IgG3 hinge region length polymorphism in Ghanaian children susceptible to and protected from clinical malaria**

| Genotype | Susceptible | | Protected | | |
|---|---|---|---|---|---|
| | No | % | no | % | p value |
| LL | 9 | 50.0 | 101 | 40.1 | |
| LM | 7 | 38.9 | 116 | 46.0 | |
| MM | 2 | 11.1 | 35 | 13.9 | |
| Chi square | | | | | 0.67 |

*L-long hinge region allele, M-medium hinge region allele*

The effect of IgG3 hinge region length on levels of antigen specific IgG3 produced was assessed and it was found that for all the antigens, the MM genotype was consistently associated with the highest antigen specific IgG3 levels while the LL genotype was associated with the lowest IgG3 levels (Figure 4.5), though not significantly different ($p = 0.142$) across the genotypes for all antigens. However, for the antigens MSP1 hybrid and GLURP-R2, the LL genotype was associated with slightly higher IgG3 levels than the LM genotype. Also, for each antigen, levels of IgG3 associated with the various hinge region genotypes were not significantly different ($p>0.05$), though for EBA-175, a trend approaching significance was observed ($p = 0.07$, Kruskal-Wallis one way ANOVA). Furthermore, the distribution of the various genotypes in three age categories (1 to 5 years, 6 to 9 years and 10 to 14 years) was examined to ascertain which genotypes dominated as the population got older. The frequency of the MM genotype was the lowest in the general population and it was also the least (5.6%) present in the older individuals of the population while the LM was the highest (54.7%) (Figure 4.6). However, for the various age groups (1 to 5 years, 6 to 9 years and 10 to 14 years), the difference was not statistically significant ($p = 0.053$; 0.076 and 0.148; One-Sample t-test) respectively.

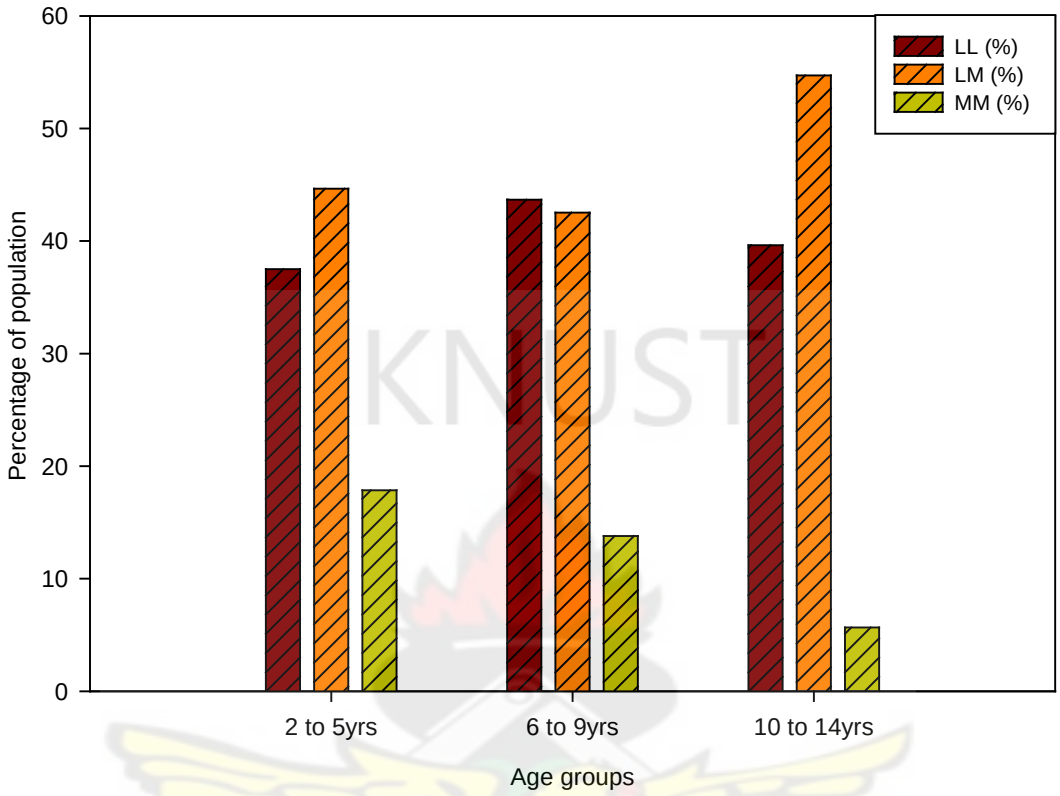



**Figure 4.6 Percentage distributions of IgG3 hinge region genotypes among various age groups of the study population.**

## 6.11. The ABO blood group system and clinical malaria

The distribution of the ABO blood group was found to be significantly different between the susceptible group and those apparently protected from clinical malaria in cohort 2 (P=0.02, $\chi^2$ analysis) (Table 4.11). The blood group O was the most represented (45.8%, n=196) among those apparently protected followed by B (31.3%, n=134) and then A (18.9%, n=81) while the AB group was the least found among the protected individuals (4.0%, n=17) (Table 4.11). The association of blood group AB with susceptibility to clinical malaria was found to be statistically

significant (P=0.02, $\chi^2$ analysis) when subjects were grouped into only two groups, AB and non AB blood groups. In cohort 1, the distribution of the ABO blood groups among individuals who were susceptible and those apparently protected from clinical malaria did not differ significantly (P=0.73, $\chi^2$ analysis) (Table 4.11) in contrast to what was observed in cohort 2. The O blood group; the most predominant blood group in the cohort was nearly equally distributed among susceptible and protected individuals (46.8% and 46.6% respectively), while only 5.9% of the blood group B individuals were protected against clinical malaria. When the population was grouped on the bases of blood group AB and non-AB, the chi-squared test still returned a non-significant difference between number of susceptible and protected individuals within the population cohort 1. Since its been shown (Cserti and Dzik 2007) that there is a higher blood group O-to-A ratio in malaria endemic populations, this ratio was estimated for both cohorts and was found to be 2.37 and 2.54 for cohorts 1 and 2 respectively (Table 4.1b).

**Table 4.11 ABO blood group distributions among individuals apparently protected and those susceptible to *P. falciparum* malaria in two community based cohort studies.**

| | **Cohort 1** | | | | | **Cohort 2** | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Susceptible | | Protected | | p-value | Susceptible | | Protected | | p-value |
| Blood group | No. | % | No. | % | | No. | % | No. | % | |
| A | 9 | 15.5 | 123 | 20.1 | | 1 | 3.3 | 81 | 18.9 | |
| B | 17 | 29.3 | 166 | 27.2 | | 12 | 40.0 | 134 | 31.3 | |
| AB | 5 | 8.6 | 36 | 5.9 | | 4 | 13.3 | 17 | 4.0 | |
| O | 27 | 46.6 | 286 | 46.8 | | 13 | 43.3 | 196 | 45.8 | |
| **Chi square** | | | | | **0.73** | | | | | **0.02** |

# CHAPTER FIVE

## 7.0 DISCUSSION

### 7.1. Study population and malaria transmission

In this study which was aimed at investigating both immunological and genetics factors which correlate with protection against clinical malaria, two community based cohorts spanning two consecutive malaria transmission seasons and a hospital based case-control study were used (Table 4.1a and Table 4.1b). The rate of clinical malaria incidence was lower than expected in the area of the community based study during the study period for both cohorts 1 and 2 but was, however, higher in the first cohort (cohort 1) than the second (cohort 2) (Table 4.1b). The first cohort consisted of children up to the age of 12 years while the second included individuals as old as 29 years. Generally, immunity to malaria is known to develop gradually with age with younger individuals of malaria endemic population being at a higher risk of clinical malaria than older ones (Pratt-Riccio *et al.,* 2005; Dodoo *et al.,* 2008). Thus, a greater proportion of participants in the first cohort were within the high risk group of clinical malaria than in the second cohort and this might explain why the malaria incidence rate was higher in the first cohort than the second. Interestingly, though a greater proportion of participants used insecticide treated net (ITN) in the first cohort than in the second, the incidence of clinical malaria was still higher in the first cohort (Table 4.1b). This could probably be because bed nets are used when children are sleeping indoors and infectious bites may be acquired at some time before children get under bed nets. This could happen if children are made to spend longer hours outside or even indoors (but not under

bed-nets) in the night when they can be vulnerable to mosquito bites. Effective therapeutics for parasite control and the coverage of the provision of ITNs for vector control are rapidly changing the natural risk of parasite exposure through vector control and thus, altering the epidemiology of malaria infection and risk of disease in many parts of Africa including Ghana (Fegan *et al.,* 2007; Okiro *et al.,* 2007; Bhattarai *et al.,* 2007; Ndugwa *et al.,* 2008; O'Meara *et al.,* 2008). This could explain the unexpectedly low rate of clinical malaria incidence observed in both cohorts.



### 7.2. Levels of naturally occurring antigen-specific antibodies in the study population

In many malaria sero-epidemiological studies, high titres of antibodies to antigens are often associated with protection (John *et al.,* 2005; Nebie *et al.,* 2008) though others have argued that antibody quantity does not necessarily mean antibody quality with respect to protection (Druilhe and Khusmith 1987; Marsh *et al.,* 1989; Bouharoun-Tayoun and Druilhe 1992a). Also, one major parameter that is assessed in potential vaccine candidate antigens in preclinical studies and during vaccine trials is their immunogenicity which is the ability of the antigen to substantially elicit a strong immune response upon encountering host immune mechanisms (Theisen *et al.,* 1994; Cavanagh *et al.,* 1998; Malkin *et al.,* 2005; John *et al.,* 2005; Villard *et al.,* 2007; Agak *et al.,* 2008; Osier *et al.,* 2008; Nebie *et al.,* 2008). Since the ELISA method used in this study was designed to allow for a direct head-to-

head comparison of antibody levels to various antigens, levels of IgG and IgM antibodies for all the antigens were compared to ascertain their levels in the natural population. IgG and IgM were chosen as parameters for comparison because these are the antibodies implicated in protective immunity and primary immune response respectively (McGREGOR *et al.,* 1963; Sabchareon *et al.,* 1991; John *et al.,* 2005; Nebie *et al.,* 2008). The data shows IgG and IgM antibodies specific to AMA1-FVO were the highest in the population studied followed by EBA-175 (Figure 4.1) and these were the only antigens in which IgG responses were always higher than IgM a finding consistent with a previous study (John *et al.,* 2005). Although literature for a head-to-head comparison of all the eight antigens used in this study is extremely rare, there are publications in which at least some of them have been tested in the same study. High prevalence of IgG to EBA-175 has been reported in Gambian children living in a seasonal malaria transmission region (Okenu *et al.,* 2000). Also, in a holoendemic malaria region in Kenya, John *et al.,* (2005), found IgG levels to EBA-175 to be higher than to AMA1 but lower IgM levels to EBA-175 than to AMA1. These findings are contrary to the IgG and IgM patterns for these antigens found in this study. Nebie *et al.,* (2008), found that in a malaria hyper-endemic region of Burkina-Faso, where malaria transmission is seasonal the geometric mean IgG levels to these antigens increased according to the order MSP1, MSP3, GLURP and AMA1 (Figure 4.1). This finding is consistent with the pattern of IgG response to these antigens in the present study. Of the two GLURP antigens tested, IgG levels to GLURP-R2 which has been described as the more immunodominant antigen (Theisen *et al.,* 1998; Oeuvray *et al.,* 2000) was clearly higher, in agreement with

previous studies of these two antigens (Pratt-Riccio *et al.,* 2005). The synthetic peptides, LR146 and AS202.11 responses were mainly of IgM antibodies and showed the least IgG responses of all the antigens tested (Figure 4.1). The general trend of higher IgM responses than IgG (except for AMA1 and EBA-175) could probably be due to the observation that, although IgM has a lower affinity for antigens compared with IgG isotypes, IgM displays increased avidity because of its pentameric structure hence the elevated responses seen. This could also explain why the antibody responses to the peptide antigens were predominantly of IgM and very low IgG. Several factors could account for the discrepancies in the levels of naturally occurring antibodies to these antigens found in the different studies. These include differences in the intensity and pattern of malaria transmission, the period in which samples were obtained and the age stratifications of the study populations, as well as differences in the ELISA protocols used in measuring antibody levels.

### 7.2.1. Pattern of IgG1-4 subclasses response

As was expected, IgG1-4 subclasses responses to most of the antigens increased with age as have been found in other studies (Nebie *et al.,* 2008; Dodoo *et al.,* 2008). Data from previous studies have shown that MSP3, GLURP and MSP1 contain B-cell epitopes that are targeted by cytophilic IgGs such as IgG1 and IgG3 (Bouharoun-Tayoun and Druilhe 1992b; Metzger *et al.,* 2003). IgG1 and IgG3 have also been reported by two other studies (Nebie *et al.,* 2008; Dodoo *et al.,* 2008) to be the main IgG subclass response to these antigens. The present study found the

IgG1 response to be the most predominant IgG subclass for the antigens AMA1, MSP3, GLURP-R2 and EBA-175; however, there was a surprisingly higher IgG2 levels than IgG3 for all the antigens except MSP3 (Figure 4.2) and this has not been observed by others (Theisen *et al.,* 1998; Nebie *et al.,* 2008; Dodoo *et al.,* 2008). One of the criteria for selecting a potentially good vaccine candidate antigen has been the ability of the antigen to elicit antibody responses capable of parasite growth inhibition in *in vitro* functional assays mostly through antibody dependent cellular inhibition (ADCI) (Theisen *et al.,* 1998; Malkin *et al.,* 2005). Cytophilic antibodies are thought to be the major mediators of ADCI since they are more efficient at recruiting phagocytic cells such as monocytes and macrophages through their Fc receptors. On the other hand, non-cytophilic antibodies such as IgG2 and IgG4 have been reported by some investigators as inhibitors of the bridging of merozoites to human monocytes by cytophilic antibodies against the same antigenic target and thereby reducing the ability of the latter to control parasite multiplication via the ADCI mechanism (Bouharoun-Tayoun and Druilhe 1992a). Thus, antigens eliciting strong cytophilic IgG responses are traditionally, often considered better vaccine candidates. However, higher IgG2 than IgG3 levels have been reported in the adult Fulanis of Eastern Sudan where malaria is hypoendemic (Nasr *et al.,* 2009) and in region of low seasonal malaria transmission in Burkina-Faso (Aucan *et al.,* 2000). In both of these populations, high IgG2 levels were associated with protection and there was a high prevalence of the 131H allele of FcγRIIA which has been shown to efficiently bind to IgG2 opsonised parasites. The present study also had very low malaria transmission similar to hypo-to-mesoendemic transmission

patterns and taken together, these findings suggest that in areas where malaria transmission is low, there is a high prevalence of IgG2 than IgG3 levels to most malaria antigens and in association with 131H-FcγRIIA could confer protection against clinical malaria. This could probably be due to the rapid clearance of IgG3 antibodies from circulation implying that, such a short-lived antibody (Cavanagh *et al.,* 2001) might require a more continuous exposure to the parasite to be maintained at significantly higher levels than IgG2. Published studies (Modiano *et al.,* 1999a; 1999b) have demonstrated the existence of marked differences in *P. falciparum* rates, malaria morbidity, and antibody responses to various *P. falciparum* antigens, and genetic background, which probably implies genetic regulation of the immune responses.

### 7.2.2. Antigen-specific antibody response with age

The levels of total IgG and subclasses to all the antigens increased with age (Figure 4.3a), reflecting cumulative exposure to malaria parasites and probably, gradual maturation of the immune system over time (Baird 1995). These findings are consistent with those from earlier studies of blood-stage malaria antigens performed in malaria endemic areas (Marsh *et al.,* 1989; Lunel and Druilhe 1989; Metzger *et al.,* 2003). In this study (cohort 2) which enrolled individuals from 1 to 29 years of age, a trend of increasing IgM levels with age was observed for all the antigens, except that, it appears IgM levels decline after age 15 years and this was true for all the antigens including the peptide antigens, LR146 and AS202.11 (Figure 4.3b).

This could mean that the mechanism of induction of antibodies to these peptide antigens might be similar to that of the recombinant antigens. In the Kilifi district of Kenya, a study involving children up to 10 years old, found higher antibody levels to these peptides to be associated with increasing age and the presence of parasitaemia at the time of sampling (Agak *et al.,* 2008). This may be explained by the fact that these peptides are weak immunogens and a constant booster with parasitaemia is required to maintain high levels of antibodies to them. It may also be that these peptides are not inducing long term memory responses and thus, primary responses typified by high IgM levels are common than IgG responses. However, this Kenyan study used a polyvalent detection antibody in the ELISA, and thus, may have measured antibody responses representing a broader spectrum of antibodies compared with the IgG and IgM specific detection antibodies used in the present study. This together with differences in intensity of malaria transmission may account for the differences in the antibody levels to these peptides measured in the two studies. Interestingly, the current study found that after the age of 6 years, IgG levels to LR146, AS202.11 and MSP1-hybrid did not vary significantly in the population studied; implying that, probably maximal IgG levels to these antigens are acquired at a younger age and these may be short lived in the absence of parasitaemia. It also appears that the highest levels of IgG to AMA1-FVO and EBA-175 are reached after the age of 10 years (Figure 4.3a) and it is then maintained suggesting that these antigens may be inducing longer term memory responses upon relatively fewer encounters with the parasite. Dodoo *et al.,* (2008) found IgM levels to GLURP, MSP3, AMA1 and MSP1 to increase with age in Ghanaian children

aged 3 to 10 years but a study in children up to age 15 years in Burkina-Faso only found an increase in IgM levels with age for GLURP and MSP1 but not MSP3 and AMA1 (Nebie *et al.,* 2008). Findings from these studies together with the present study seem to suggest that in young individuals perhaps up to the age of 15 years, who are in an active phase of acquiring immunity to malaria, IgM responses may be crucial but not in semi-immune adults living in malaria endemic areas. It also suggests that malaria immunity in adults (individuals beyond 15 years old) might be more dependent on IgG responses than IgM.



### 7.3. Hemoglobin levels and acute malaria parasitaemia

It has been observed that in malaria-endemic areas, the incidence of severe anaemia and age -specific rates of anaemia strongly correlate with the intensity of *Plasmodium falciparum* transmission (Clark and Tomlison 1949), and that significant improvements in hematological indices have been seen after malaria control trials (Greenwood *et al.,* 1989; Alonso *et al.,* 1991). While there may be several causes for the anaemia, *P. falciparum* infection is believed to be a major contributory factor to the etiology of severe anaemia seen in malaria endemic areas (Koram *et al.,* 2000). In this present study and during acute malaria, hemoglobin levels correlated positively with age while parasitaemia on the other hand negatively correlated with age in the two cohorts as well as the hospital based case-control study (Figure 4.4). The increase in Hb levels with age could be as a result of maturity of the erythropoietic system with age while the lower density parasitaemia

associated with acute malaria in older individuals could perhaps be explained by a more matured innate immune system in older individuals. In addition, cumulative exposure to the parasite with age, evidenced by the high antibody titres in older individuals might explain how older individuals are able to keep parasite loads low. However, in the hospital based study, high density parasitaemia did not necessarily imply disease severity, confirming reports that severity of malaria might depend more on other factors such as age as well as host and parasite genetics than parasite density during acute malaria (Frodsham and Hill 2004; Milner *et al.,* 2008).



### 7.4. Malaria specific antibody levels and clinical malaria

Associations between levels of malaria specific antibodies and clinical malaria have been reported by many investigators with the cytophilic IgG antibodies being the most implicated in protection against malaria (Okenu *et al.,* 2000; Pratt-Riccio *et al.,* 2005; Osier *et al.,* 2008; Nebie *et al.,* 2008; Dodoo *et al.,* 2008). However, though baseline antibody levels were always higher in individuals 'protected' against than those 'susceptible' to clinical malaria, the differences were not statistically significant for any of the antigens tested (Table 4.2 and Table 4.3). The general lack of statistically significant association of any of these antibodies with protection is however, indicative of the extremely low incidence rate of malaria found in the study population during the period of the study, thus making malaria a rare disease and rendering the study statistically under-powered to detect significant associations with protection. Participants were kept under weekly active and passive

malaria surveillance and were presumptively treated with artesunate-amodiaquine combination therapy when they had fever as consistent with the current clinical practice; a practice which might have contributed to reducing malaria incidence and hence, the statistical power of the study. An attempt to correct for presumptive drug treatment as a potential source of bias was made by excluding individuals who reported sick at the health facilities and treated with the artesunate-amodiaquine combination therapy but failed to meet the parasitaemia threshold of ≥2500 used in the analysis. Such individuals were classified as 'indeterminate' with regards to malaria susceptibility or protection. In spite of this correction, the study could still not found statistically significant associations with the antibody levels and protection. Exposure to malaria is an important potential confounder in sero-epidemiological studies and inadequate measurement and adjustment for differences in exposure may lead to underestimation of the strength of associations between immunological variables and malaria incidence (Nebie *et al.,* 2008). However, the present study found trends of decreasing risk of clinical malaria with high levels of IgG1 and IgG4 to MSP3 as well as IgG3 to both GLURP R0 and EBA-175 approaching significance (Table 4.2 and Table 4.3). Several studies in different populations have identified IgG3 to GLURP R0 to be associated with protection from clinical malaria (Bouharoun-Tayoun and Druilhe 1992b; Oeuvray *et al.,* 2000; Soe *et al.,* 2004; Nebie *et al.,* 2008; Dodoo *et al.,* 2008) and this antibody has also been demonstrated to inhibit *P. falciparum* growth in *in vitro* functional assays but only in coorporation with monocytes in ADCI (Theisen *et al.,* 1998). High levels of IgG3 to EBA-175 were not found to be associated with reduced risk of clinical

malaria in a malaria holoendemic region of Kenya (Ohas *et al.,* 2004) but Okenu *et al.,* (2000), found trends toward protection against clinical malaria with high levels of IgG to EBA-175 in a region of seasonal malaria transmission in the Gambia. Also protection associated with high levels of IgG3 but not IgG1 or IgG4 to MSP3 have been reported (Soe *et al.,* 2004; Roussilhon *et al.,* 2007; Segeja *et al.,* 2010). The trend towards significant association between the levels of non-cytophilic IgG4 against MSP3 and clinical protection is in contrast with the observation that, non cytophilic antibodies can inhibit the bridging of merozoites to human monocytes by cytophilic antibodies against the same antigen resulting in the failure of the latter to control parasite multiplication by the ADCI mechanism (Bouharoun-Tayoun and Druilhe 1992b). Also, it has been shown that non-cytophilic IgG4 antibodies against blood stage antigens such as GLURP, MSP1, MSP2 and RESA are associated with enhanced risk of infection and with a high risk of malaria attack (Aucan *et al.,* 2000). All the same, one cannot exclude the possibility that this subclass may be an indicator of a specific cytokine response critical for protection against clinical malaria (Nebie *et al.,* 2008). There have been studies which reported a genetic linkage of parasitemia to chromosome 5q31-q33 which contains genes encoding cytokines involved in isotype switching toward IgG4 and in proliferation, differentiation, and activation of immune system cells (Metcalf 1991; Rihet *et al.,* 1998; Chomarat and Banchereau 1998). IgG4 to GLURP was found to be protective in children (6 month to 15 years) in Burkina-faso (Nebie *et al.,* 2008), however, as was found in the present study, IgG4 levels measured in the Burkinabès were low also, suggesting the need for further investigations into the exact role of

this antibody in protection against malaria. IgG1 and IgG3 subclasses have been shown to be effective mediators of antibody-dependent cellular inhibition of *P. falciparum*-infected erythrocytes (Bouharoun-Tayoun and Druilhe 1992b; Theisen *et al.,* 1998) and have also been found to be the subclasses capable of mediating phagocytosis through opsonisation (Bouharoun-Tayoun *et al.,* 1995). This is because IgG1 and IgG3 bind to Fcγ receptors on monocytes, macrophages, and neutrophils, which are the cells involved in antibody-dependent cellular inhibition and phagocytosis.



### 7.5. Distribution of FcγII and FcγIII receptor polymorphisms in the study population

Fcγ receptors serve as a crucial link between the humoral and cellular arms of immune responses. They are mainly involved in activation (except FcγIIB) of accessory cells against pathogens and thus making these receptors very essential in the host defense against infection. The frequencies of FcγIIA-131H/R and FcγIIB-232I/T genotypes in the present study were found to be significantly different from those predicted by the Hardy-Weinberg equilibrium (Table 4.4) suggesting that these genes may be under some form of selective pressure within the study population. A previous study in Thai adult malaria patients (Omi *et al.,* 2002) found FcγIIA-131H/R to be in Hardy Weinberg equilibrium contrary to the findings in this study. This discrepancy could be due to differences in the ethnic backgrounds as well as the differences in malaria transmission patterns and intensity of the

populations studied since reports have shown that genotype frequencies for this polymorphism differ in different ethnic groups (Nasr *et al.,* 2008; Israelsson *et al.,* 2008). The non-random distribution of allelic variations in FcγRIIA, FcγRIIB and FcγRIIIA among the study population (Table 4.5 and Table 4.6) suggests that certain allelic combinations might perhaps be more crucial in reducing the risk of clinical malaria than others. On the other hand it could also be that the alleles are distributed in such a way that those that are disadvantageous to survival are compensated for by preferentially linking to those alleles that confer protection. In this study, the VH haplotype was found to be linked to FcγRIIB-232 I, whereas the RF haplotype was preferentially linked to FcγRIIB-232 T (Table 4.6). The FcγRIIIA-176V allele has a stronger affinity for IgG3 than the FcγRIIIA-176F, FcγRIIA-131H is unique in its efficient binding to IgG2 in addition to IgG1 and IgG3 while the FcγRIIB-232T allele has been shown to decrease the inhibitory effect of FcγRIIB on phagocytosis and superoxide production in macrophages and on proliferation in B cells *in vitro* (Floto *et al.,* 2005). Results from many immuno-epidemiological studies have shown that total IgG3 or antigen-specific IgG3 is more superior to IgG1 in protection against malaria and this has also been confirmed in *in vitro* parasite growth inhibition assays such as the ADCI assay (Oeuvray *et al.,* 2000; Soe *et al.,* 2004; Jafarshad *et al.,* 2007). In addition, it is also thought that IgG1 could down-regulate monocyte activation by triggering the regulatory FcγRIIB (Nimmerjahn and Ravetch 2005). Thus, the preferential allelic distribution in the present study population seem to always link potent activators (VH haplotypes) with the more potent inhibitor (FcγRIIB-232I), while the weaker

activators (Tishkoff *et al.,* 2001) are linked to the weaker inhibitory receptor (FcγRIIB-232T) perhaps to maintain a balance in the strength of immune response elicited.

### 7.6. Association of Fcγ receptor genotypes with clinical malaria

The present study found statistically significant association between FcγRIIIB genotypes and protection against clinical malaria but not for the other Fcγ-receptor polymorphisms studied (Table 4.7). This is contrary to reports from previous studies (Ouma *et al.,* 2006; Sinha *et al.,* 2008). On the other hand, Omi *et al.,* (2002) also found no association between FcγRIII-176V/F genotypes and clinical malaria in adult Thai malaria patients though an earlier study by the same group reported an association between severe malaria and FcγRIIA and FcγRIIIB allotypes (Omi *et al.,* 2002). FcγRIIB-232TT frequency found in the present study was 11.5% and it has been suggested that this allele has been maintained in malaria endemic populations due to its protective effect against clinical malaria (Niederer *et al.,* 2010) thou its be found to predispose individuals to systemic lopus erythematosus (SLE) (Molokhia and McKeigue 2000). A study found higher levels of antimalarial antibodies and TNF-α in FcγRIIB-deficient mice (Clatworthy *et al.,* 2007). In the same study, a human monocyte line transfected with FcγRIIB-232T or primary human monocyte-derived macrophages from FcγRIIB-232T homozygous donors showed increased phagocytosis of opsonized parasitized erythrocytes compared to FcγRIIB-232I homozygous controls. However, further work is necessary to

establish the association between this allele (FcγRIIB-232T) and protection against clinical malaria in cohort studies. The present study also found a significant association between both severe and uncomplicated malaria and the FcγRIIIB-NA2/NA2 genotype (Table 4.8) which is consistent with findings by Omi *et al.*, (2002) where this same genotype was associated with susceptibility to cerebral malaria. On the contrary, the FcγRIIIB-NA1/NA1 genotype was significantly associated with protection against both severe and uncomplicated clinical malaria (Table 4.8). FcγRIIIB-NA2 shows more glycosylation than FcγRIIIB-NA1 resulting in a decreased affinity of the former for IgG3 compared with the latter (Niederer *et al.,* 2010). Since IgG3 is more potent in parasite clearance compared with IgG1, a decreased affinity for IgG3 in the FcγRIIIB-NA2 might correspond to a decreased IgG1 and IgG3-mediated phagocytosis in neutrophils in the absence of complement (Salmon *et al.,* 1990; Bredius *et al.,* 1994) and this may account for its association with clinical malaria pathogenesis. On the other hand the FcγRIIIB-NA1 allele was found to be consistently associated with protection against both uncomplicated and severe forms of malaria probably due to enhanced phagocytosis in the presence of this allele. This study also found some individuals lacking the FcγRIIIB genes (HNA-null individuals) as have been reported elsewhere (Kissel *et al.,* 2000) but the current data did not suggest that this allele has any impact on clinical malaria.

## 7.7. IgG3 hinge region length polymorphism and clinical malaria

The L-allele and LM genotype for IgG3 hinge region had the highest allelic and genotypic frequencies respectively in the sub-population studied (Table 4.9). IgG3 has been shown to be superior over IgG1 in parasite clearance, a property which has been 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 in the former than the later (Redpath *et al.,* 1998; Oeuvray *et al.,* 2000; Soe *et al.,* 2004; Jafarshad *et al.,* 2007). In the same vein, it might be true that these features of IgG3 are more pronounced in polymorphs with longer hinge region than those with medium or even shorter hinge region, thus, making those with longer hinge region even more efficient at parasite clearance. However, in this present study, the MM genotype which was the least found in the population was always associated with the highest IgG3 levels while the LL genotype was almost always consistent with the lowest IgG3 levels (Figure 4.5) in agreement with observations of Theisen *et al.,* (unpublished data). Though the differences in the genotype distributions among susceptible and protected individuals were not statistically significant due to loss of power as a result of fewer clinical malaria cases in the cohort, the association of long (LL) and medium (MM) hinge regions with low and high IgG3 levels respectively might be important in malaria immunity. Thus, it appears the less efficient MM allotype probably compensates for its limitations by having more IgG3 produced and this might explain why the LM genotype was the highest found among individuals 'protected' against clinical malaria. This is because the LM can benefit from both the highly efficient IgG3 L-allele as well as the probably less

efficient but high levels of M-allele IgG3 during parasite clearance. This finding will be very critical in the accurate interpretation of data from pre-clinical studies and clinical trials since IgG3 hinge region length appears to be an important confounder which must be corrected for in these studies. However, this hypothesis will warrant further testing in a larger population where malaria is more endemic to clearly ascertain the extent to which the different IgG3 hinge region polymorphs contribute to naturally acquired immunity against clinical malaria. In addition, a functional assay such as growth inhibition or the ADCI assay involving purified IgG3 from individuals with known IgG3 hinge region genotype will be necessary to confirm the superiority of one polymorph over the other during parasite clearance. Interestingly, the current data seem to suggest that, as the population gets older, a consistency of decreasing MM genotypic frequency begins to emerge (Figure 4.6) with the frequency being higher among younger individuals of the population than older ones. The reason for this trend could be multi-factorial; however, this pattern of genotype distribution seems to imply a case of heterozygous advantage similar to what is seen for the sickle-cell trait though a probably less severe in this instance. Though the genotypic frequencies did not deviate significantly from those expected from the Hardy-Weinberg equilibrium, it appears less MM genotype individuals survive to reproductive age to pass on their genes to the next generation, a feature which suggests a gene pool under selective pressure. This could probably mean that, the selective pressure which could as well be some factor other than malaria has become constant leading to the establishment of equilibrium in the IgG3 hinge region genotypic distribution in the population. However, this will also need to be

tested in a larger population to clearly evaluate the consequence of IgG3 hinge region genotype on survival of individuals in a malaria endemic population.

## 7.8. ABO blood group antigens and malaria transmission

The relationship between the ABO blood group antigens and malaria was suggested more than 40 years ago when Athreya and Coriell observed in 1967 that the global distribution of the ABO blood groups in humans showed a trend consistent with an effect which is under the selective pressure of *P. falciparum* (Athreya and Coriell 1967). Geographic regions where malaria is currently endemic or was previously endemic tend to have a higher group O-to-A ratio than regions where malaria has not been endemic (Uneke *et al.,* 2006). It has also been hypothesized that during an infection with *P. falciparum,* blood group O offers a survival advantage, group-A confers a disadvantage and group B has an intermediate effect (Cserti and Dzik 2007). In this study, blood group O was the most dominant blood type and a higher blood group O-to-A ratio (>2.0) was observed in both cohorts (Table 4.1b), a finding which is consistent with the what has been reported for Ghana (Cserti and Dzik 2007). Blood group O was of the highest proportion among individuals apparently protected from clinical malaria followed by the B group in both cohorts but was statistically significant only in cohort 2 (Table 4.11). This suggests that blood group O may confer protection against clinical malaria. Several studies have reported significant association between the ABO blood groups and total malaria cases (Pant *et al.,* 1992); lower frequency of *P. falciparum* parasitaemia in group A

and O (Singh *et al.,* 1995) as well as association of blood group A with rosetting and severe malaria (Lell *et al.,* 1999). The O group has also been associated with protection against high density parasitaemia (Migot-Nabias *et al.,* 2000) and severe malaria (Pathirana *et al.,* 2005) in a longitudinal and case-control study respectively. Conversely, some investigators have found no significant association between the ABO blood groups and *P. falciparum* malaria prevalence (Bayoumi *et al.,* 1986; Montoya *et al.,* 1994; Uneke *et al.,* 2006), parasitaemia (Martin *et al.,* 1979; Kassim and Ejezie 1982), and parasitaemia or parasite antibody titer (Akinboye and Ogunrinade 1987; Thakur and Verma 1992). Thus, besides the pattern of geographical distribution of the ABO blood groups which seem to support an association with *P. falciparum*, findings from the various studies that investigated the relationship between the ABO blood group and malaria prevalence, parasitaemia, antibody titer, and number of episodes have been inconsistent and contradictory. The apparent discrepancies in these studies may reflect the complex interaction between *P. falciparum* malaria and the host immune responses (Uneke 2007). Thus, it appears the association between *P. falciparum* malaria and the ABO blood groups is still quite debatable and further work might be necessary to clearly resolve these discrepancies.

# CHAPTER SIX

## 7.0 CONCLUSION AND RECOMMENDATION

### 7.1 Conclusion

The study has implicated IgG3 to GLURP-R0 and IgG4 to MSP3-FVO to be important in protection against clinical malaria suggesting a multivalent vaccine made from these antigens could be effective in conferring immunity against clinical malaria. Blood groups O and B were found to be more advantageous in protecting against clinical malaria than groups A and AB in the population studied while the FcγRIIIB-NA1/NA1 and FcγRIIIB-NA2/NA2 genotypes were significantly associated with protection against and susceptibility to clinical malaria respectively. IgG3 hinge region length genotype was also found to influence IgG3 levels in a complementary manner where the highest and the lowest IgG3 levels were found to be associated with the MM and LL genotypes respectively. In addition, an overrepresentation of the LM genotype was found in individuals apparently protected against clinical malaria suggesting a possible case of heterozygous advantage evidenced by the underrepresentation of the MM genotype in older members of the population. However, the present study was underpowered to predict statistically significant difference between some of the parameters studied due to the unexpectedly low malaria cases recorded during the study period.

## 7.2 Recommendation

1. Future studies should be conducted with more individuals in the high malaria risk age groups such that the events recorded will be substantial enough to enable the study have sufficient statistical power to predict statistically significant trends. Alternatively, in such an area where malaria incidence has become low, a case-control study where a malaria episode is a necessary inclusion criterion for enrolment into the study might be more appropriate than a longitudinal cohort.

2. *In vitro* functional studies such as the growth inhibition assay or the ADCI assay involving the antibodies and FcγRs purportedly associated with protection against clinical malaria are also recommended to critically evaluate the synergistic or individual functionality of these antibodies and FcγRs in malaria immunity. Also functional assays involving purified IgG3 from individuals with known IgG3 hinge region genotype will be necessary to confirm the superiority or otherwise of one polymorph over the other during parasite clearance. In addition, a large population size study will be necessary to confirm the demographic distribution of IgG3 hinge region length polymorphism in a malaria endemic population and its influence on the outcome of *P. falciparum* infection as well as levels of IgG3 antibodies produced. Such a study will also enable the hypothesis of heterozygous advantage of IgG3 hinge region length genotype to be tested with a greater degree of certainty. Taken together, all these will enable a more reliable interpretation of data from malaria vaccine trials and from preclinical studies.

# Appendices

## Appendix I

### Preparation of standard solutions and buffers

Unless otherwise stated, all standard solutions for ELISAs were prepared with double distilled water (dd $H_2O$), while water (Milli-Q) in PCRs solutions were prepared with Millipore filtered water autoclaved autoclaved at 121 lb/sq for 15 minutes in an Eyela autoclave (Rikikkaki, Tokyo).

### Primers for PCR

Dry oligos used in PCR reaction were reconstituted and while already reconstituted primers from manufacturer (DNA Technology A/S, Risskov, Denmark) were diluted to working concentrations using PCR grade water.

### 5X Orange G (Gel loading buffer)

20% (w/ v) Ficoll, 25 mM EDTA, 2.5% (w/ v) orange G and stored at room temperature.

### DNA molecular weight size markers

DirectLoad 50bp DNA molecular weight marker was obtained from the manufacturers (Sigma Missouri, USA).

**NEBuffer 4**

Supplied by manufacturer (New England Biolabs, USA)

**PCR Buffers**

HotStar buffer I and TemPASE buffer I supplied by manufacturer (Amplicon, Hamburg, Germany)



**Agarose gel**

To prepare a 2%w/v agarose gel, 4.0g of agarose powder (SeaKem®GTG® Agarose, Lonza, Rockland, ME, USA) was put into flask and X1 TAE was added to make a volume of 200ml. It was heated in microwave oven (230V, 50Hz, 2660W, 12.0A) for 3minute to dissolve. Four drops (1 drop/g agarose) of Ethidium Bromide (AppliChem, Damstadt, Germany) was added. The gel was cast to set in a chamber with comb to make the wells. In preparing 3%w/v 6g agarose powder was used added to 200ml X1 TAE buffer.

**Coating buffer (Plain PBS, pH 7.04)**

This was prepared by dissolving 1 PBS tablet in 500.0ml double distilled water.

**Blocking Buffer (PBS with 5 % milkpowder, 0.1% Tween-20)**

In preparing 1.0L, 2 tablets of PBS were added to a beaker containing 1000.0ml deionised water and placed the flask on a magnetic stirrer without heating and stir until all was in solution. 50.0 g of skimmed milk was then added together with 1 ml of Tween-20 and stirred until all were in solution.

**Serum Dilution Buffer (PBS with 2.5 % milkpowder, 0.1% Tween-20 and 0.02% Na-azide)**

In order to prepare 1000.0ml of the serum dilution buffer, 2 PBS tablets were added to a beaker containing 1000.0ml deionised water and placed the flask on a magnetic stirrer without heating and stirred until all was dissolved. 25.0 g of skimmed milk, 1.0 ml of Tween-20 and 2.0 ml of 10 % Na-azide solution were added and the solution stirred until a homogeneous mixture was obtained. The 10 % Na-azide solution was prepared by adding 40.0ml of deionised water to 4.0g of Na-azide.

**Dilution Buffer (PBS with 2.5 % milkpowder and 0.1% Tween-20)**

To prepare 1000.0ml of dilution buffer, 2 tablets of PBS were added to a beaker containing 1000.0ml deionised water and placed on a magnetic stirrer without heating and stirred until all was in solution. 25.0 g of skimmed milk and 1.0 ml of Tween-20 were added and the solution stirred until all were dissolved.

**Washing Buffer (PBS with 0.1% Tween-20 and 0.5 M NaCl)**

To prepare 5000.0ml washing buffer, 10 tablets of PBS were added to a beaker containing 5000.0 ml deionised water and placed on a magnetic stirrer without heating and stirred until all is in solution. 146.25g of NaCl and 5.0ml of Tween-20 and solution stirred until all had dissolved.

**Colour Solution [TMB (3, 3', 5, 5'-Tetramethylbenzidine)]**

The substrate solution, ready to use 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.

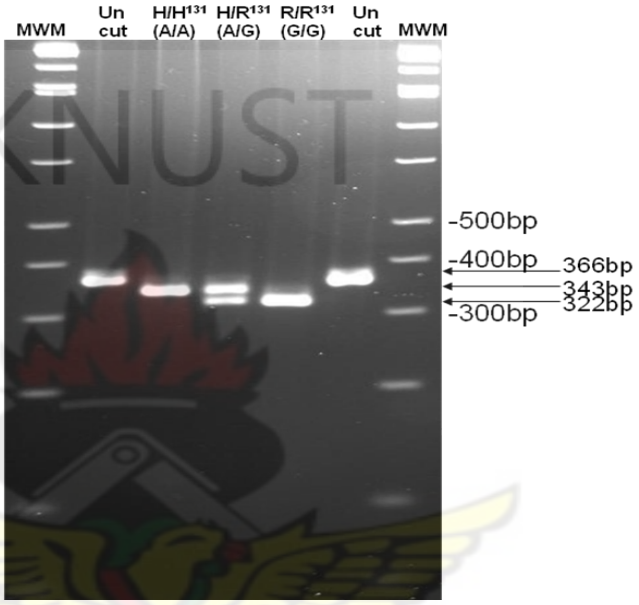
**Stop Solution (0.2M $H_2SO_4$)**

To prepare 1000.0ml of stop solution, 20.0ml of 10.0M $H_2SO_4$ was added to 980.0ml of deionised water and the solution shaken to mix. It was then cooled to room temperature and kept in the hood until required.

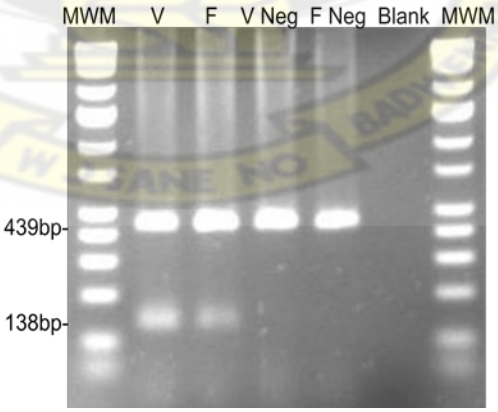
**Appendix II**

Agarose gel photographs with a 50bp molecular weight DNA marker and pyrograms for the various polymorphisms.

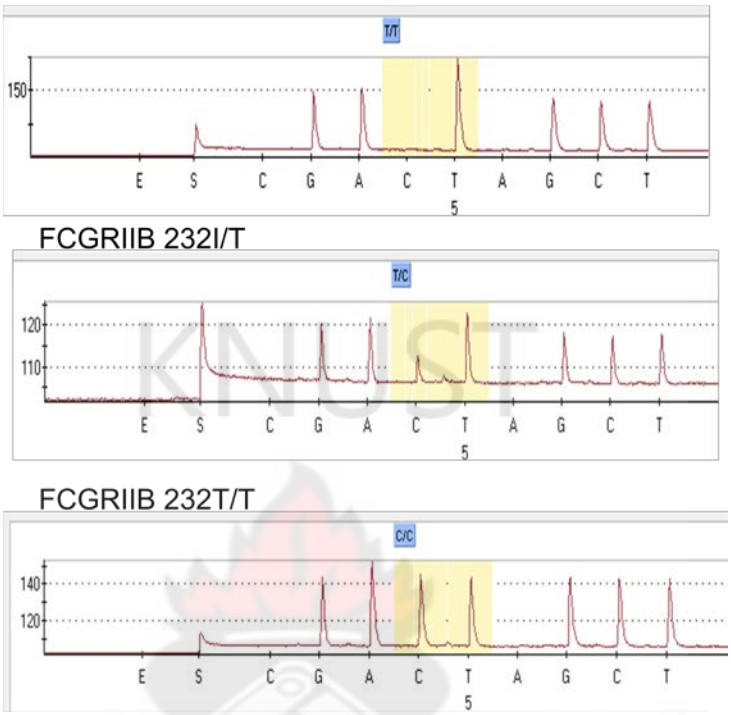
a. FCGR2A- 131H/R PCR and *Bst*UI Digestion Products



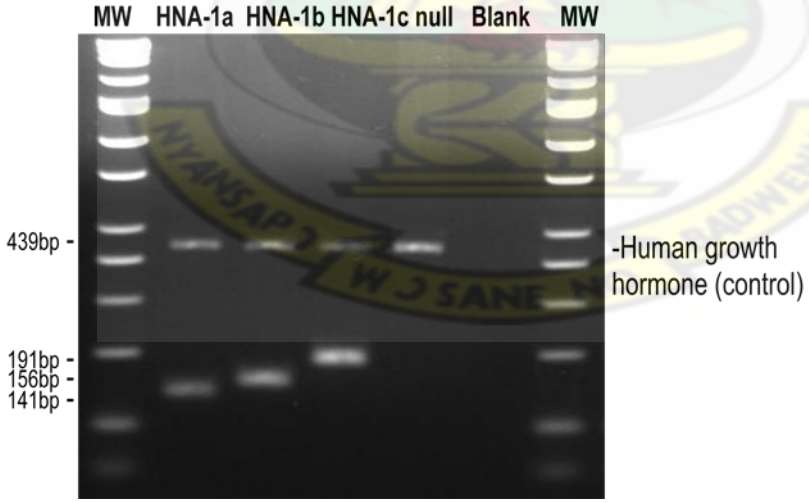

b. Gel Picture of FCGR3A 158F/V Allotypes



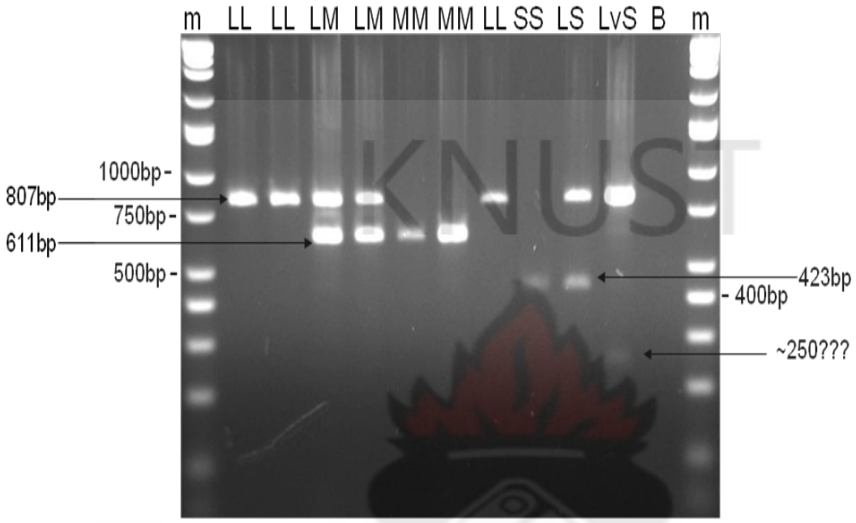
c. FCGR2B 232T/C or FCGRIIB 232I/T SNP Pyrograms




**d.** Gel Picture of the FCGR3B Allotypes



e. IgG3 Hinge Region Genotypes



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