From the Department of Microbiology, Tumor and Cell Biology (MTC)
Karolinska Institutet, Stockholm, Sweden

FUNCTIONAL ANTIBODY RESPONSES TO THE *PLASMODIUM FALCIPARUM* MEROZOITE

Hodan Ahmed Ismail

Karolinska Institutet

Stockholm 2014

All previously published papers were reproduced with permission from the publisher.

Published by Karolinska Institutet. Printed by Universitetsservice US-AB

© Hodan Ahmed Ismail, 2014
ISBN 978-91-7549-429-6

To my loving parents

ABSTRACT

Plasmodium falciparum is a leading cause of death among children under the age of five and pregnant women in sub-Saharan Africa. More than one third of the world's population is at risk of contracting malaria, and 70 % of the cases are found in sub-Saharan Africa. Emerging drug resistance in parasites and limited effect of vector control calls for an effective vaccine. It is known that individuals living in malaria endemic countries develop naturally acquired immunity after repeated exposure. Antibodies are important components of acquired immunity, and it has been shown that passive transfer of antibodies from immune donors to individuals with *P. falciparum* infections reduced parasitemia and clinical symptoms. Antibodies against several *P. falciparum* merozoite antigens have been found to be associated with protective immunity. It is of great importance to understand the underlying functional role of antibodies in the development of protective immunity against severe malaria.

In a cross-sectional study in Uganda, the quantitative and qualitative differences in antibody responses to a panel of merozoite antigens in children with uncomplicated or severe malaria were evaluated using a set of assays including ELISA, Invasion Inhibition Assays (IIA), NH_4SCN-ELISA and Surface Plasmon Resonance (SPR). Children with uncomplicated malaria had higher antibody levels against PfEBA-181, MSP2-Fc27, MSP2-3D7 and PfAMA1 when compared to children with severe malaria. Acquired antibodies against PfRh2 and PfAMA1 in ELISA correlated with invasion inhibition of two clinical isolates in IIA, and anti-PfAMA1-antibodies in ELISA correlated with increased anti-PfAMA1-antibody affinity in SPR. Importantly, the only assay that correlated with initial parasitemia in the children was the IIA. Both MSP2-Fc27 and MSP2-3D7 allelic variants were present in both children groups, but there was a higher number of genotypes in uncomplicated malaria compared to in children with severe malaria.

P. falciparum clinical isolates collected from Ugandan children with uncomplicated malaria or severe malaria were further investigated for rosetting, parasite multiplication and RBC invasion. Optimal *in vitro* growth conditions were established, which allowed for phenotypic studies of clinical *P. falciparum* isolates. Presence of serum in growth cultures was found to be essential for optimal surface presentation of PfEMP1 and maintenance of rosettes. Higher peripheral parasitemia, higher rosetting levels and higher multiplication rates were observed in children with severe malaria and these correlated positively with one another. Rosetting might enhance successful merozoite invasion *in vivo*, hence could be the reason it is found to be associated with severe disease. Furthermore, parasite invasion into trypsin- and chymotrypsin-treated RBC differed between the uncomplicated and severe groups, and isolates from children with uncomplicated malaria showed higher sensitivity to enzyme treatment. The majority of clinical isolates used a sialic acid independent invasion pathway. Parasite invasion is central to parasite replication and virulence, and it is essential to know which invasion pathways are used for vaccine studies.

Naturally acquired antibody responses to *P. falciparum* merozoite antigens was further studied in a longitudinal study over almost one year in children and adults from Nigeria. The malaria protective effects of the hemoglobin S (HbAS) allele were also investigated. In both children and adults, the antibody response against PfEBA175 was more prominent than that against PfRh2, and cytophilic IgG1 and IgG3 against PfEBA175 were the predominant antibodies even though we could also see some response for IgG2 and IgG4. Individuals with higher total IgG responses against both PfEBA175 and PfRh2 had lower parasitemia over the course of the study period. Furthermore, children with HbAS had higher antibody responses against merozoite antigens compared to adults, and this might have protective effects against malaria.

In conclusion this thesis emphasizes the great importance of using a combination of functional assays, such as SPR and IIA, to study the functional role of acquired antibodies in the development of protective immunological responses against severe malaria. Furthermore, investigations of parasite invasion and rosetting in the context of pathogenesis of severe malaria are crucial as both are important for parasite replication and virulence. Taken together, future vaccine studies should include functional assays that would allow the investigation of differences in immunological responses against severe and uncomplicated malaria. Also, to consider the protective effects of red blood polymorphisms and their role in acquired immunity in the populations, would be of great value for future vaccine studies.

LIST OF PUBLICATIONS

This thesis is based on the following papers, which will be referred in the text by their Roman numbers.

I. **Ahmed Ismail H**, Ribacke U, Reiling L, Normark J, Egwang T, Kironde F, Beeson JG, Wahlgren, Persson KEM.
Acquired antibodies to merozoite antigens in children from Uganda with uncomplicated or severe *Plasmodium falciparum* malaria.
Clin Vaccine Immunol. 2013: 8: 1170-80

II. Ribacke U, Moll K, Albrecht L, **Ahmed Ismail H**, Normark J, Flaberg E, Szekely L, Hultenby K, Persson KEM, Egwang TG, Wahlgren M.
Improved *in vitro* culture of *Plasmodium falciparum* permits establishments of clinical isolates with preserved multiplication, invasion and resetting phenotypes.
PloS One. 2013: e69781

III. **Ahmed Ismail H**, Muyideen Kolapo T, Langer C, Reiling L, Beeson JG, Wahlgren M, Nwuba R, Persson KEM.
Antibody responses against EBA175 and PfRh2 in naturally acquired immunity against *Plasmodium falciparum* malaria.
Manuscript

TABLE OF CONTENTS

1 INTRODUCTION 1
1.1 PRESENT MALARIA BURDEN 1
1.2 APICOMPLEXAN PROTOZOA 1
1.2.1 PLASMODIUM SPECIES 2
1.2.2 LIFE CYCLE 2
1.3 DISEASE CHARACTERISTICS 4
1.3.1 SEVERE MALARIA 4
1.3.2 SEVERE ANAEMIA 5
1.3.3 ACUTE RESPIRATORY DISTRESS SYNDROME 5
1.3.4 CEREBRAL MALARIA 6
1.3.5 PREGNANCY-ASSOCIATED MALARIA 6
1.4 *PLASMODIUM FALCIPARUM* PATHOGENESIS 7
1.4.1 HOST REMODELING 7
1.4.2 PROTEIN TRAFFICKING 8
1.4.3 ANTIGENIC VARIATION IN PLASMODIUM FALCIPARUM 8
1.4.4 SEQUESTRATION 10
1.5 *PLASMODIUM FALCIPARUM* MEROZOITE INVASION 13
1.5.1 EGRESS OF MEROZOITES 13
1.5.2 INITIAL ATTACHMENT 14
1.5.3 MEROZOITE REORIENTATION AND IRREVERSIBLE TIGHT JUNCTION FORMATION 14
1.5.4 MEROZOITE ENTRY 15
1.6 SIALIC ACID PATHWAYS 16
1.7 *PLASMODIUM FALCIPARUM* MEROZOITE ANTIGENS 17
1.7.1 APICAL MEMBRANE ANTIGEN 1 (AMA-1) 17
1.7.2 MEROZOITE SURFACE PROTEIN 1 18
1.7.3 MEROZOITE SURFACE PROTEIN 2 19
1.7.4 ERYTHROCTYE BINDING LIKE (EBL) PROTEIN FAMILY 19
1.7.5 RETICULOCYTE BINDING PROTEIN HOMOLOGUE (Rh) FAMILY 21
1.8 NATURALLY ACQUIRED IMMUNITY TO MALARIA 22
1.8.1 IMMUNOGLOBULIN M (IgM), IgG AND SUBCLASS IgG1-4 IN MALARIA 24
1.8.2 ANTIBODY RESPONSES TO THE P. FALCIPARUM MEROZOITE ANTIGENS 24
1.9 HUMAN RED BLOOD CELL POLYMORPHIMS AND MALARIA 27
1.9.1 MALARIA PROTECTION BY HbAS 27
1.10 MALARIA VACCINES 28

2 SCOPE OF THE THESIS 30

3 EXPERIMENTAL PROCEDURES 31
3.1 STUDY POPULATIONS 31
3.1.1 UGANDA (PAPER I and II) 31
3.1.2 NIGERIA (PAPER III) 31
3.2 ETHICAL CONSIDERATION 31
3.3 PARASITES AND *IN VITRO* CULTURES CONDITIONS 32
3.4 INVASION INHIBITION ASSAY 32

3.5 INVASION INTO ENZYME TREATED RED BLOOD CELLS 32
3.6 RECOMBINANT MEROZOITE ANTIGENS 33
3.7 MEASURING TOTAL IMMUNOGLOBULIN G (IGG) AGAINGST RECOMBINANT MEROZOITE ANTIGENS BY ELISA 33
3.8 MEASURING SUBCLASS IG1-IGG4 AGAINGST RECOMBINANT MEROZOITE ANTIGENS BY ELISA 33
3.9 SURFACE PLASMON RESONANCE 34
3.10 AMMONIUM THIOCYANATE (NH_4SCN)-ELISA 34
3.11 DETERMINATION OF *P.FALCIPARUM* GENETIC DIVERSITY 34

4 RESULTS AND DISCUSSION 35
4.1 PAPER I 35
4.2 PAPER II 37
4.3 PAPER III 39

5 CONCLUDING REMARKS AND FUTURE DIRECTIONS 41

6 ACKNOWLEDGEMENTS 43

7 REFERENCES 46

LIST OF ABBREVIATIONS

AMA1	Apical membrane antigen 1
CM	Cerebral malaria
CR1	Complement receptor 1
CSA	Chondroitin-sulfate A
CSP	Circumsporozoite protein
EBA	Erythrocyte binding antigen
GlyA	Glycophorin A
GPI	Glycosylphosphatidylinositol
HbAS	Hemoglobin AS
IgG	Immunoglobulin G
ITN	Insecticide-treated bed net
MC	Mauer´s cleft
MOI	Multiplicity of infection
MSP	Merozoite surface protein
PAM	Pregnancy-associated malaria
Pf	*Plasmodium falciparum*
PfEMP1	Plasmodium falciparum erythrocyte membrane protein 1
pRBC	parasitized red blood cells
PVM	Parasitophorous vacuolar membrane
RBC	Red blood cell
Rh	Reticulocyte binding homologue protein
RON	Rhoptry neck protein
SERA	Serine repeat antigen
spp	Species
TNF	Tumor necrosis factor
TRAP	Thrombospondin-related anonymous protein
ups	Upstream sequence
var	Gene encoding PfEMP1
WHO	World Health Organization

Gene names are written in italics and lowercase letters (e.g. *var* gene). Protein names are written in capital letters (e.g. VAR2CSA).

1 INTRODUCTION

1.1 PRESENT MALARIA BURDEN

Plasmodium falciparum malaria is a major global health issue. More than one third of the world's population is at risk of contracting malaria, and up to 70 % of the cases are found in sub-Saharan Africa (Figure 1). There were around 219 million cases and 660 000 deaths in 2010, mainly children under the age of five and pregnant women (WHO, 2012). It is estimated that a child dies from malaria every 40-th second, resulting in approximately 2 000 lives lost daily around the world (Sachs, 2002).

Malaria mortality rates are the highest in countries with the lowest Gross National Income (GNI) (WHO, 2012). The prevention and treatment, and loss of income due to malaria morbidity or mortality have a great toll on a poor agriculture household (Malaney et al., 2004). Population movement into regions of high malaria transmission, changes in agriculture, deforesting and long-term climate changes are risk factors that can have an effect on the emerging malaria pattern (Sachs and Malaney, 2002).

The coverage of vector control intervention has increased in the past decade in sub-Saharan Africa. The households owning insecticide-treated bed nets (ITN) have increased from 5 % in 2000 to 53 % in 2012 (WHO, 2012). Implementation of control programs using drug treatment of malaria infected individuals, preventive drug treatment of populations at risk, mosquito control with ITN and indoor insecticide spraying have contributed to a decrease of malaria morbidity and mortality (Kappe et al., 2010; WHO, 2012). Between 2000 and 2010, the global malaria incidence decreased by 17 % and mortality by 26 % (WHO, 2011).

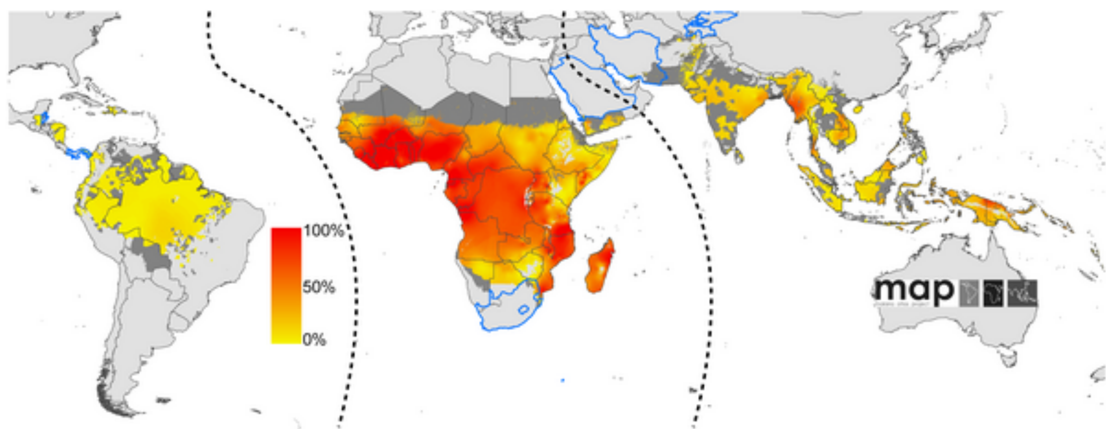

Figure 1. Global limits and endemicity of *Plasmodium falciparum* as estimated in 2007. (Adapted from (Hay et al., 2010)).

1.2 APICOMPLEXAN PROTOZOA

Apicomplexan protozoa is a very large group including 4 000 - 5 000 species that both infect invertebrates and vertebrates. Apicomplexan protozoa are unicellular organisms characterized by unique organelles, apicoplast and apical complex structures, such as mirconemes and rhoptries located at the apical end of the cells. These organelles have a key

role in the invasion of animal cells. *Plasmodium spp* belong to the apicomplexan phylum. Other medical important parasites in this phylum include *Toxoplasma spp* and *Cryptosporidium spp*, and in agriculture there are *Theileria spp*, *Babesia spp*, and *Eimeria spp*. Species in this group have complex life cycles and are transmitted by insect vectors (Murray, 2009).

1.2.1 *PLASMODIUM* SPECIES

Plasmodium spp. consists of more than a hundred species that infect a wide range of animals, monkeys, birds and rodents. At least 5 species are known to infect humans: *Plasmodium falciparum, Plasmodium vivax, Plasmodium ovale, Plasmoidum malariae* and *Plasmodium knowlesi*. Globally, *P. falciparum* cause the deadliest form of malaria and is the major cause of morbidity and mortality. *P. vivax* is mainly found in South America and Asia, and is a major cause of morbidity outside Africa (Anstey et al., 2012; Price et al., 2007). *P. vivax* was historically described as " benign tertian malaria", but this term is misleading since *P. vivax* has been reported to cause major morbidity and in worst case mortality (Barcus et al., 2007; Price et al., 2009). *P. malariae* and *P. ovale* cause a relatively mild form of malaria and are less frequent to infect humans. *P. knowlesi* was recently recognized as a fifth malaria species and was found to cause malaria in macaques but also proven to infect humans (White, 2008).

The scope of this thesis is the *P. falciparum* and the antibody responses to *P. falciparum* merozoite antigens.

1.2.2 LIFE CYCLE

The *Plasmodium spp*. life cycle involves a mosquito vector and a vertebrate animal, with asexual and sexual developmental stages (Figure 2).

The host life cycle consists of the pre-erythrocytic stage and the erythrocytic stage. In the pre-erythrocytic stage, the female *Anopheles* mosquito injects sporozoites (harboured in the salivary glands) into the dermis of the host during a blood meal. Real time imaging in animal studies using GFP expressing *P. berghei* parasites showed that 20 sporozoites were injected under the dermis and had a robust forward gliding locomotion movement (Amino et al., 2006). The sporozoites eventually interact with the blood vessels in the dermis through gliding movements, and reach the blood circulation and lymphatic vessels, before entering the liver circulation. The sporozoites invading the lymphatic vessels are eventually drained in the lymphatic node (Amino et al., 2006). The gliding movement used by the sporozoites is essential and allows them to migrate through cells, and to pass through biological barriers, the cytoplasma of host cells (Amino et al., 2008; Frevert et al., 2005). The sporozoites express surface antigens, such as circumsporozoite protein (CSP) that has been shown to have a vital role in the sporozoite development, motility, invasion and subversion of hepatocytes (Frevert and Nardin, 2008; Kappe et al., 2004; Singh et al., 2007). Once the sporozoites have reached the liver circulation, it takes only minutes before they reach the liver. By using the gliding movement, the sporozoites migrate through the Kuppfer cells lining and several hepatocytes, before invading a heaptocyte and forming a parasitophorous vacuole (PV) (Baer et al., 2007b; Mota et al., 2002; Mota et al., 2001; Pradel and Frevert, 2001). During a time period of around 6-15 days depending on *Plasmodium spp*., the sporozoites undergo differentiation and replicates into thousands of haploid forms called merozoites per hepatocyte cell (Baer et al., 2007a; Tarun et al., 2006). The merozoites exit the hepatocyte and re-enter the blood circulation.

This stage is referred to as the erythrocytic stage. The free merozoites invade red blood cells (RBC), and replication takes place through asexual forming and releasing of new merozoites from rupturing RBC. The parasitized RBC (pRBC) develops into ring form, trophozoite, and finally a mature schizont (this process is known as schizogony), which after mitotic nuclear division bursts and releases 8-16 new merozoites into the blood circulation. This stage takes 24-72 hours (depending on *Plasmodium spp)*. The formation of a parasitophorous vacuolar membrane (PVM) is essential for the parasite's survival and growth. It is formed during the invasion process and surrounds the parasite during the intra-erythrocytic development and is critical for structural and transport functions (Boddey and Cowman, 2013; Cooke et al., 2004; Haase and de Koning-Ward, 2010). The replication of merozoites can result in thousands of pRBC in the blood circulation. The erythrocytic stage is a complex process and is responsible for the symptoms and illness caused by malaria if not treated.

Some of the pRBC in the blood circulation develop into the sexual form of the malaria parasite, female and male gametocytes. The switch may be due to a genetic response to a specific stimulus by host or parasite origin (Alano and Carter, 1990; Bruce et al., 1990; Talman et al., 2004). Furthermore, it has been shown that merozoites from a single schizont either continue the asexual cycle or develop into either female or male gametocytes (Bruce et al., 1990; Smith et al., 2000). During a blood meal, a feeding female *Anopheles* mosquito ingests female and male gametocytes. The female and male gametocytes are released into the midgut of the mosquito, and develop to female and male gametes, which fuse to form diploid zygotes. The zygote further develops into an elongated and motile ookinete. The ookinete invades the midgut wall and develops into an oocyte. The oocyte develops and matures, and finally ruptures to release sporozoites. The sporozoites migrate to the salivary gland of the mosquito, and during the next blood meal they will be injected into the human host, thus the continuation of the *Plasmodium* life cycle (Pimenta et al., 1994; Touray et al., 1992).

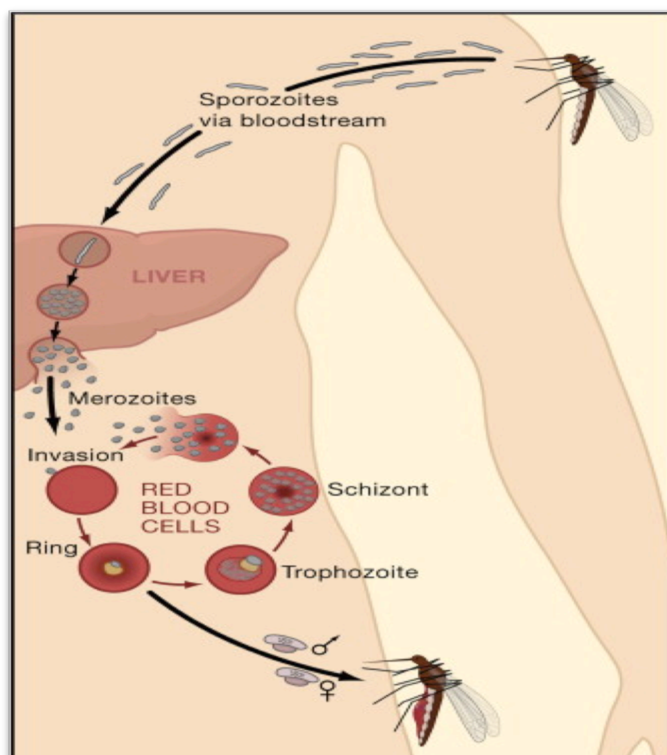

Figure 2. The Life Cycle of *P. falciparum* in the Human Host (Adapted from (Cowman and Crabb, 2006) and published with permission from Nature Publishing Group).

1.3 DISEASE CHARACTERISTICS

Malaria gives rise to a wide variety of symptoms, ranging from asymptomatic, mild to severe symptoms with high mortality rate. Malaria is categorized as uncomplicated or severe, and is described as flu like manifestations (7-15 days post infection), with the classical "cold stage", a combination of shivering and feeling cold (despite elevated body temperature) for an hour. The "hot stage" lasts for 2-6 h and includes fever, headache, vomiting, nausea, and maybe seizures in children. This is followed by the "sweating stage", with body temperature decline and malaise. At the end of this stage, the patient feels complete exhaustion and tiredness. The sudden onset of these clinical symptoms is also known as a malaria paroxysm. The clinical symptoms that are manifested during the disease are caused by the erythrocytic stage of the parasites. Febrile attacks occur periodically every 24, 48 or 72 hours (depending on the *Plasmodium spp*.). During the blood stage, the pRBC ruptures releasing fever associated substances, including hemozoin and other toxic factors, which stimulate macrophages and dendritic cells to produce proinflammatory cytokines. The secretion of high levels of tumor necrosis factor α (TNFα) into the circulation has an adverse effect on disease progression (Kern et al., 1989; Molyneux et al., 1991), and is more often found in patients with severe than uncomplicated malaria (Kwiatkowski, 1990).

P. vivax, *P. ovale* and *P. malariae* have onsets of malaria paroxysms every 48 hours (tertian) or 72 hours (quartian) (depending on the species) while *P. knowlesi* comes every 24 hours (semi-tertian). Other symptoms seen in malaria patients include hemolytic anaemia with a dramatical drop in hemogloblin during onset of disease (Jakeman et al., 1999; Lamikanra et al., 2007). Splenomegaly can be observed during the acute malaria onset, and increase in size with repeated infections (Chaves et al., 2011; Del Portillo et al., 2012). Jaundice can be due to hemolysis and seen in all types of malaria. However, *P. falciparum* has been shown to be the leading cause of malaria hepatopathy, and an increasing number of cases have been reported in Asian countries (Anand and Puri, 2005; WHO, 2000b).

During the *P. falciparum* infection, mature trophozoites sequester within the microvasculature of the brain, lung, kidney, intestines, skin and placenta, and major organs. The sequestration will be discussed in detail in section 1.4.4.

1.3.1 SEVERE MALARIA

The clinical outcomes of *P. falciparum* infection are numerous and diverse, with severe and complicated manifestations. The progression from uncomplicated malaria disease to severe malaria with fatal outcome can be rapid, within hours. Severe malaria is a number of different conditions that can appear alone or in combination. The complications and severe manifestations are severe anaemia, renal failure, acute respiratory distress syndrome (ARDS), pulmonary edema, unrousable coma (cerebral coma), multiple convulsions, circulatory collapse, renal failure, hypoglycemia, abnormal bleeding, acidosis, hyperlactemia and hyperparasitemia (WHO, 2000b). *P. falciparum* can also cause pregnancy-associated malaria (PAM), which causes complications in both the mother and infant. The mortality rate for severe malaria with treatment falls to 15-20 % (WHO, 2010).

1.3.2 SEVERE ANAEMIA

Severe anaemia is defined as having a hemogloblin level <5g/dl in children or <7g/dl in adults (WHO, 2000a) or a hematocrit below 15%. The fatality rate due to severe anaemia in malaria is lower than in ARDS and cerebral malaria (Murphy and Breman, 2001). However, together with cerebral malaria, severe anaemia is a leading cause of mortality, between 6-16% in children (Marsh et al., 1995), and 6% in pregnant woman (Shulman et al., 1996). Malaria patients have an increased risk of hemolysis (90%) compared to healthy individuals (Jakeman et al., 1999). An average of 8.5 unparasitized RBC are destroyed for every invaded RBC, resulting in anaemia due to destruction of healthy RBC and pRBC.

A study using a rodent model demonstrated that the clearance of pRBC was due to the uptake of phagocytic monocytes and macrophages, and this caused anaemia (Evans et al., 2006). Hemozoin released from rupturing pRBC has been associated with anaemia by inhibiting erythroid development, and this caused induction of apoptosis (Lamikanra et al., 2009). Macrophage ingestion of hemozoin caused macrophages to release macrophage migration inhibitor factor (MIF), which inhibits erythroid progenitor derived colony formation (Martiney et al., 2000). Malaria toxins stimulated macrophages to produce the pro-inflammatory cytokines TNF-α, INF-γ and IL-12 during the acute blood stage, and they have been shown *in vitro* and *in vivo* to mediate chronic disease, including malaria (Taverne et al., 1994). During the chronic stages of malaria, bone marrow hypoplasia and subsequent dyserythropoesis are also believed to play role in severe anaemia (Wickramasinghe and Abdalla, 2000).

1.3.3 ACUTE RESPIRATORY DISTRESS SYNDROME

There are different types of respiratory distress found in malaria patients. It is defined as a respiratory rate that reaches above 40 breaths per minute. Hyperventilation is the result of metabolic acidosis, caused by renal dysfunction and insufficient clearance of lactic acid and this cause low blood pH and an increased respiratory rate. Hyperventilation is the most common type of respiratory distress found in African children with malaria, and it is also found in adults (English et al., 1996; Marsh et al., 1995).

The acute respiratory distress syndrome (ARDS) is a severe condition associated with high risk of death, and is increasingly associated with malaria. ARDS is associated with alveolar inflammation, damage to the alveolar-capillary membrane and severe hypoxemia (Taylor et al., 2006). Malaria-associated ARDS is mainly found in adults, with mortality rates up to 80 %, even with treatment. ARDS can also occur in children, but is rare. Most cases of ARDS are found in low transmission areas or in non-immune travellers. Pregnant women with severe malaria are at higher risk to develop malaria-associated ARDS. It has been shown that the prevalence of ARDS ranges from 2 % up to 20 % in adults with severe malaria, but it depends on the study area and parasite species (Taylor et al., 2006; Taylor et al., 2012). The pathogenesis of ARDS is not fully understood. Damage to the endothelial barrier results in interstitial edema, and edema fluids leak out to the alveoli. This results in formation of eosinophilic hyaline membranes, surrounding the alveolar walls (Valecha et al., 2009). Post mortem histo-pathological studies have shown presence of monocytes and macrophages inside the blood capillaries and intersititium, and in alveolar spaces (Duarte et al., 1985). Hemozoin has also been seen in pulmonary macrophages, due to phagocytosis, causing production of

cytokines and inflammatory mediators (Valecha et al., 2009). *P. falciparum* parasites sequester within the capillaries of the lung, and are believed to be involved in the pathogenesis.

1.3.4 CEREBRAL MALARIA

The most severe neurological complication of *P. falciparum* infection is cerebral malaria (CM), and it is a major cause of acute non-traumatic encephalopathy in tropical countries. According to WHO guidelines, CM as clinical syndrome is characterized by unrousable coma (inability to respond to a painful stimuli) at least 1 hour after a seizure (scoring <3 on the Blantyre scale), positive *P. falciparum* blood smears, and exclusion of other causes of encephalopathy (Molyneux et al., 1989; WHO, 2000b). Hospital admitted children with cerebral malaria often have a history of 1-3 days of fever, vomiting, coughing and main neurological signs including coma, seizures and brainstem signs (Molyneux et al., 1989; Newton et al., 2000). Mortality due to CM is high, about 20% in children and adults, and the majority of deaths occur within 24 hours from hospital admission, before the anti-malarial drugs have had any effect. Mortality is high among children with shock, hypoglycaemia, multiple and prolonged seizures or coma (Faiz et al., 1998; Idro et al., 2004). The onset of CM in children is very rapid within a few days of fever, while in adults it develops gradually (Kochar et al., 2002; Molyneux et al., 1989). Around 60 % of children with cerebral malaria develop seizures after hospital admission (Crawley et al., 1996; Crawley et al., 2001). Seizures are thought to be an important cause of impairment for consciousness in children. Around 10% of malaria patients who survive cerebral malaria have neurological sequelae, caused by seizures, prolonged and deep coma and hypoglycaemia (Bondi, 1992; Walker et al., 1992). The majority of patients with cerebral malaria have full recovery but there has been recognized an increasing number of reported cases of brain injury, especially in African children, over the past 20 years. Long-term neurological or cognitive deficits include effect on memory, motor deficits, epilepsy, speech and language difficulties, and difficulties in concentration and attention (Ngoungou and Preux, 2008; van Hensbroek et al., 1997). Neurological sequelae are less common in adults.

Pathogenesis studies of CM have mainly been post mortem surveys, *in vitro* studies or animal studies. Sequestration of *P. falciparum* parasites in the microvasculature of the brain has been observed in several post mortem examinations of children and adults, who died from CM. Sequestration is believed to be central to the pathogenesis of CM, and extensive sequestration leads to obstruction of blood flow and causes tissue hypoxia (Pongponratn et al., 1991). The hypoxia causes metabolic exchange and release of pro-inflammatory cytokines, which are protective or inflammatory for the malaria patient. Increased levels of INF-γ and TNFα have been shown in both in human and murine studies to be implicated in the pathogenesis of CM (Hunt and Grau, 2003).

1.3.5 PREGNANCY-ASSOCIATED MALARIA

Individuals living in malaria endemic areas become semi-immune to severe malaria after repeated exposure. However, women become susceptible to severe malaria during pregnancy. The risk of contracting pregnancy-associated malaria (PAM) is highest among primigravide women, with lower incidence and severity in women of higher parity. Women living in unstable or low transmission areas have a higher risk of severe malaria, CM and respiratory distress, while women in high transmission areas more often suffer from severe anaemia (Duffy

and Fried, 2005). PAM causes infant low birth weight, prematurity, still birth, anaemia in newborns, maternal anaemia and is responsible for,100 000-250 000 infant and fetal deaths and 1 500 maternal deaths in sub-Saharan Africa each year (Duffy and Fried, 2005). During PAM, the pRBC sequester in the intervillous space of the placenta, which restricts the blood flow and nutrient passage across the placenta (Beeson et al., 1999; Fried and Duffy, 1996). There is a harmful inflammatory response, with increased infiltration of monocytes in the placenta and increased levels of pro-inflammatory cytokines (Ismail et al., 2000; Rogerson et al., 2003).

1.4 *PLASMODIUM FALCIPARUM* PATHOGENESIS

1.4.1 HOST REMODELING

The pRBC undergoes host cell remodeling to sustain the survival and development of the parasite. The host remodeling occurs as follows: I) *P. falciparum* proteins interact with the RBC cytoskeleton, and alter the cell´s mechanical properties or II) modify cell architecture of the host cell membrane or III) participate in the transport machinery responsible for protein trafficking to the RBC surface.

The main membrane structures involved in the trafficing machinery and that are also found in the RBC cytosol, are the tubulovesicular network (TVN) and Mauers clefts (MC) (Atkinson and Aikawa, 1990; Bannister and Dluzewski, 1990; Langreth et al., 1978). The TVN is the most prominent of the membrane structures and comprises an interconnected network of tubular and vesicular membranes that extends from the PV into the cytoplasma (Elmendorf and Haldar, 1994). Additionally, TVN is thought to be involved in nutrient import (Lauer et al., 1997). MCs are characterized by a translucent lumen and electron-dense coats found in the RBC cytosol, and are located close to the RBC membrane (Atkinson and Aikawa, 1990; Kriek et al., 2003). MCs are found in the cytosol of late stage rings, and recently they were found to be present from 2-6 hours post infection (Bannister et al., 2004; Langreth et al., 1978). Several exported proteins including PfEMP1, RIFIN (repetitive interspersed proteins), SURFIN (surface-associated interspersed protein) and Pf332, interact with MC and are thought to be involved in the protein transportation between PV and the RBC membrane. However, the MC transportation is not fully understood (Haeggstrom et al., 2004; Kriek et al., 2003; Wickham et al., 2001). Several integral proteins have been shown to be important for the correct MC formation. *P. falciparum* skeleton binding protein 1 (PfSBP1), and also membrane-associated histidine rich protein 1 (MAHRP1), ring exposed protein 1 (REX1) and *P. falciparum* erythrocyte membrane 3 (PfEMP3) (Maier et al., 2009b) have been shown to be of interest.

Knobs are found on the surface of the pRBC during the trophozoite and schizont development. Knobs are dense electron protrusions and have been shown to serve as sequestration attachments during sequestration in the microvasculature (Aikawa, 1988b). Electron microscopy studies of patients who died from CM showed pRBC attached to the endothelium via knobs (Aikawa, 1988a). The *P. falciparum* knob-associated histidine-rich protein (KAHRP) is essential for knob formation, and found on the cytoplasmic side of RBC. KAHRP interacts with spectrin, actin and ankyrin and anchor PfEMP1 to the cytoskeleton of RBCs (Crabb et al., 1997; Culvenor et al., 1987; Oh et al., 2000; Waller et al., 1999). Gene disruption of KAHRP has demonstrated impaired cytoadhesion, hence suggesting the importance of KAHRP as essential for PfEMP1 surface expression (Crabb et al., 1997).

KAHRP has been shown to interact with the ATS domain of PfEMP1 (Waller et al., 1999), and also to interact with spectrin and actin implying a stabilizing function (Kilejian et al., 1991).

1.4.2 PROTEIN TRAFFICKING

The RBC lacks the secretory apparatus found in other nucleated cells, and the parasite can't use host mechanisms for protein transport and export and it must establish a *de novo* secretory system within the host cell cytoplasm. The protein transport system is complex, involving sorting and signalling events inside the mature pRBC. The parasite proteins need to reach the RBC cytosol, and to do so they have to cross two membranes; the parasite plasma membrane (PM) and parasitophorous vacuolar membrane (PVM) that encloses the parasite, then cross into to the host cell cytosol. Major routes for protein transport are the secretory and endocytic pathways in eukaryotic cells. Key components such as the endoplasmatic reticulum (ER) and the Golgi apparatus are required for the classical secretory pathway. Secreted proteins are co-translationally inserted into ER, and then directed from ER to the Golgi, endosomes and lysosomes by the anterograde secretory pathway. The parasites have a range of unique organelles fed by secretory pathways including food vacuole, apicoplast and three secretory granules essential for merozoite invasion (rhoptories, dense granules and micromeres) (Waller et al., 2000; Wickham et al., 2001).

Proteins that are transported to the surface contain a conserved amino acid motif known as the *Plasmodium* export element (PEXEL) (Marti et al., 2004), or also called the vacuolar transport signal (VTS) (Hiller et al., 2004). The motif is conserved in all *Plasmodium spp*. Around 200-400 soluble and membrane bound proteins carry the motif, and are known as *P. falciparum* exportome (Sargeant et al., 2006; van Ooij et al., 2008). PfEMP1 is one of the most important and well-characterized export proteins in *P. falciparum*. PfEMP1 proteins are over 300 kDa, and do not have a signal sequence but have a C-terminal trans-membrane domain that is important for the entry into the ER (Knuepfer et al., 2005). PfEMP1 has a conserved N-terminal sequence, with partial homology to the PEXEL motif. However, the PfEMP1 PEXEL-like motif is not cleaved by plasmepsin V, suggesting it is functionally distinct (Goldberg and Cowman, 2010). PfSBP1 is required for the localization of PfEMP1 into the surface of RBCs (Cooke et al., 2006; Maier et al., 2007). It has been reported that gene disruption of PfSBP1 and membrane-associated histidine-rich-protein-1 (MAHRP1) will limit the export of PfEMP1 to the RBC surface (Cooke et al., 2006; Maier et al., 2007; Spycher et al., 2008). This shows the essential role of MC in the protein trafficking.

1.4.3 ANTIGENIC VARIATION IN *PLASMODIUM FALCIPARUM*

Many pathogens have developed an ability known as antigenic variation to avoid immune recognition. Antigenic variation involves a coordinated expression of variant surface antigens to evade the host immune response, establish infection and transmission to new host. Recurrence of infection caused by a pathogen indicates a pathogen with sophisticated immune evasive mechanisms. Antigenic variation is found in a selection of infectious pathogens including *P. falciparum*, *Trypanosoma brucei*, *Borrelia hermsii* and *Candida spp* (Hoyer, 2001; Smith et al., 1995; Turner, 1999). Antigenic variation in *P. falciparum* was shown *in vivo* using squirrel monkeys for experimental blood stage infections (Hommel et al., 1983). Below the surface antigens involved in antigenic variation by *P. falciparum* are discussed.

1.4.3.1 SURFACE ANTIGENS OF P. FALCIPARUM: PfEMP1

PfEMP1 is a major adhesion protein responsible for rosetting and sequestration of the pRBC in the deep vasculature (Chen et al., 1998). PfEMP1 is encoded by a highly diverse *var* gene family that undergoes antigenic variation and generates diverse adhesive proteins (Baruch et al., 1996; Smith et al., 1995; Su et al., 1995). The *var* genes have a two exon-structure with total length of 8-14 kb, and there are approximately 60 *var* genes per parasite genome located mainly in the highly polymorphic subtelomeric regions and central parts of the 14 chromosomes (Gardner et al., 2002; Su et al., 1995). The singly expressed *var* gene determines the antigenic, cytoadherence and virulence phenotype. This phenomenon is known as mutually exclusive expression, and studies have shown that it is regulated on a translational level independent of antigen production (Dzikowski et al., 2006; Voss et al., 2006).

PfEMP1 consists of multi-domains (200-350 kDa) and the first exon encodes for a protein with a hypervariable extracellular binding part composed of a variable N-terminal segment (NTS), various Duffy binding-like domains (DBL), and cysteine rich interdomain (CIDR) with interspersed C2 interdomains. The second exon encodes for a conserved intracellular acid terminal segment (ATS) (Lavstsen et al., 2003; Smith et al., 2000; Su et al., 1995). The domains are involved in different binding specificities for host receptors (Baruch et al., 1996; Bir et al., 2006; Smith et al., 2000). PfEMP1 proteins across parasite isolates have shown a significant sequence variation, contributing to the repertoire of PfEMP1 variants. The high level of sequence diversity in the *var* gene family is due to the gene conversion and recombination (Flick and Chen, 2004; Freitas-Junior et al., 2000). The chromosomal location and transcription orientation of the *var* gene family have been shown to resemble the similarities in the 5´upstream of *ups*. *var* genes are classified into three major groups (A, B, C) and two intermediate groups (B/A and B/C) based on the four main upstream sequences (Ups); UpsA, UpsB, UpsC, and Ups E. The previously known UpsD is grouped with UpsA (Bull et al., 2005; Kraemer et al., 2007; Lavstsen et al., 2003). *var* genes in rosetting parasites belong to group A, while both groups A and B are often transcribed in severe malaria patients (Bull et al., 2005; Jensen et al., 2004; Normark et al., 2007).

varcsa1 (also termed as *varCOMMON*), *var2csa* and *var 3* are *var* genes shown to be conserved in sequenced parasite isolates, and they do not fit the classification described above. *var1csa* is flanked by UpsA2 and transcribed in the majority of all clinical isolates from different geographical regions, and has an atypical transcription pattern throughout the blood stage life cycle. *var2csa* is highly conserved and is flanked by 5´UpsE and shown to be strongly linked with mediating PAM through sequestration of pRBC to CSA in the placenta (Kyes et al., 1999; Lavstsen et al., 2003; Rowe et al., 2002a). *var3* is smallest known *var* gene, and groups within the upsA. It has been found in isolates from different geographic regions (Gardner et al., 2002; Trimnell et al., 2006).

The extracellular domains of PfEMP1 generate diverse adhesive proteins to different domains. For example Duffy binding like-1 (DBL1) have shown to bind CR1, blood group A and heparan sulfate on endothelial cells and RBCs (Barragan et al., 2000 a; Barragan et al., 2000 b; Rowe et al., 1997; Vogt et al., 2003). CIDR1 has been shown to bind to CD36 and IgM (Baruch et al., 1996; Chattopadhyay et al., 2004).

1.4.3.2 OTHER SURFACE ANTIGENS

Besides PfEMP1 there are other parasite proteins that have been suggested to be exposed on the surface of pRBC. There are the *rif* genes encoding for the repetitive interspersed protein (RIFINs) family, which have been shown to be expressed on the surface of pRBC. The highly polymorphic *rif* genes are composed of two exons with a subtelomeric location, centrometric to the *var* genes (Kyes et al., 1999). The exons encode for the 30-45 kDa RIFIN proteins, and the proteins are expressed in a clone dependent manner. The *rif* gene family holds 160 gene copies in the genome, and can be subdivided into two sub classes, group A and group B (Gardner et al., 2002; Joannin et al., 2008), which differ from one another by 25 amino acids in the semi-conserved domain. Group A appears to be exported into the host cell via Maurer´s cleft, whereas group B accumulate inside the parasite (Khattab and Klinkert, 2006; Petter et al., 2007). However, the function of the RIFINs is unclear, but is thought to be involved in the immune evasion due to the high gene copy number. It has been observed that asymptomatic children have higher antibody titers against RIFINs compared to children with severe malaria (Abdel-Latif et al., 2003; Abdel-Latif et al., 2002). Another study showed that high titers of antibodies against recombinant RIFIN in children with cerebral malaria were not protective (Schreiber et al., 2006). The presence of RIFINs has been demonstrated in *P. falciparum* gametocytes as well as in the asexual stages, but the function remains speculative (Petter et al., 2008). The RIFIN family is clustered together with the *var* and the subtelomeric variable open reading frame (*stevor*).

The STEVOR proteins are around 30-40 kDa, and less polymorphic than the RIFIN proteins. There are approximately around 30-40 copies per genome found in all chromosomes, thus making them the third largest gene family in *P. falciparum*. STEVORs have been shown to be transcribed during the asexual and sexual life cycle stages (Cheng et al., 1998; Florens et al., 2002; Sutherland, 2001), thus maybe contributing to the survival of the parasite. Clinical isolates have been show to express STEVORs more than laboratory strains. Studies have shown STEVOR proteins to be localized inside the MC, and on the pRBC membrane (Blythe et al., 2008; Kaviratne et al., 2002; Lavazec et al., 2006; Niang et al., 2009). STEVORs might have a role in merozoite invasion, since they have been detected in the apical end of merozoites and in the rhoptries (Blythe et al., 2008; Khattab and Klinkert, 2006; Khattab and Meri, 2011).

The surface associated interspersed (SURFIN) gene family consists of 10 *surf* gene members, located within or close to the subtelomers in five out of the 14 chromosomes (Gardner et al., 2002). The high molecular SURFIN proteins are related to the *P. vivax* transmembrane protein, PvSTP1 (*Plasmodium vivax* subtelomeric transmembrane protein 1) and the VIR protein (of the *P.vivax*). Studies have shown that SURFIN proteins might be involved in merozoite invasion, and they are found in the MC and the surface of pRBC (Mphande et al., 2008; Winter et al., 2005).

1.4.4 SEQUESTRATION

During the *P. falciparum* infection only the early stages are seen in the peripheral blood circulation. The modifications of the pRBC are due to parasite-derived proteins transported to the surface of the pRBC, To avoid clearance by the spleen, the mature pRBC sequester in different organs. Sequestration is either adherence to endothelial cells lining of the blood vessels (cytoadhesion) or by binding to unparasitizied RBC (rosetting).

1.4.4.1 CYTOADHESION

The term cytoadhesion is the ability of *P. falciparum* pRBC to adhere to vascular endothelium. This is seen in several organs such as brain, intestine, liver, lung, skin and the syncytiotrophoblast cell lining of the placenta. The cytoadhesion in the microvasculature not only protects the mature pRBC from splenic clearance, but also provides a relative hypoxic environment for parasite proliferation and RBC invasion.

P. falciparum has evolved to bind to receptors on different cell-types, and a number of endothelial receptors have been identified as targets for pRBC. The endothelial receptors include cluster of differentiation (CD36), intracellular adhesion molecule-1 (ICAM-1), chondroitin-sulfate A (CSA), thrombospondin (TSP), vascular cell adhesion molecule 1 (VCAM-1) and E-selectin.

CD36 is a glycoprotein that is widely expressed on the microvascular endothelium, and is also found on monocytes, macrophages, dendritic cells and platelets (Silverstein and Febbraio, 2009). CD36 binding has been shown to be a common adhesion phenotype by clinical *P. falciparum* isolates, in static and flow conditions *in vitro* (Cooke et al., 1994; Hasler et al., 1990; Newbold et al., 1997; Ockenhouse et al., 1991). CD36 is a frequent target of isolates from patients with both uncomplicated and severe malaria (Turner et al., 1994).

ICAM-1 is an essential cell adhesion molecule involved in inflammation and immunity. Several tissues and cells, including endothelial cells, monocytes and lymphocytes, express ICAM-1. Soluble ICAM-1 is found in plasma, or released by cell surface ICAM-1 in response to inflammatory cytokines or endothelial damage (Lawson and Wolf, 2009). The receptor is one of several adhesion molecules important in *P. falciparum* malaria. Expression of ICAM-1 can be both beneficial and harmful to the pRBC. The surface expression of ICAM-1 is required for INF- γ response of natural killer cells to pRBC (Baratin et al., 2007). Direct adhesion of pRBC to ICAM-1 on cerebral endothelial cells may contribute to cerebral malaria. Post mortem studies of brain tissue from patients who died from cerebral malaria demonstrated adhesion of pRBC, platelets, and leukocytes to brain endothelium, and were associated with increased ICAM-1 expression (Silamut et al., 1999). Laboratory studies have shown that *in vitro* CD36 binding was more common among cerebral malaria patients (Newbold et al., 1997). A study from Kenya showed a correlation between cerebral malaria and ICAM-1 binding under flow conditions (Ochola et al., 2011). Earlier studies of ICAM-1 binding in clinical isolates in static assays did not show any significant correlation with severe malaria disease (Heddini et al., 2004; Newbold et al., 1997; Rogerson et al., 1999).

Chondroitin-sulfate A (CSA) is a glycosamino-glycan and is a main receptor involved in PAM. CSA is found on the syncytiotrophoblasts in the placenta. Placental parasites bind to CSA but not to CD36, while non placental parasites rarely bind to CSA (Rogerson et al., 2007). IgG in serum from multigravid women living in malaria endemic areas are able to block CSA binding (Beeson et al., 1999; Fried and Duffy, 1996; Maubert et al., 2000). Now it is well established that the adhesion of pRBC to CSA depends on a particular PfEMP1 variant called VAR2CSA (Salanti et al., 2004; Salanti et al., 2003). Hyalonic acid (HA) is another receptor on the placenta, and a majority of placenta parasites have been shown to bind both CSA and HA (Beeson et al., 2000; Rasti et al., 2006). Additionally, non-immune immunoglobulins bridge pRBCs to syncytiotrophoblasts (Flick et al., 2001; Rasti et al., 2006).

1.4.4.2 ROSETTING

The rosetting phenomena was first observed in laboratory strains *in vitro*, but also *ex vivo* in fresh clinical isolates from malaria infected individuals (Carlson et al., 1990; Udomsangpetch et al., 1989; Wahlgren et al., 1992). Rosetting is defined as one mature trophozoite-stage pRBC binding to two or more unparasitized RBCs (Figure 3), and are apparent 16-18-h post infection and remain throughtout parasite maturation and schizogony up until they rupture (Treutiger et al., 1992). Rosetting parasites causing obstruction in the microvasculature of the brain and other organs are believed to contribute to the severity of the disease, causing blockage of blood flow resulting in hypoxia and tissue damage. Studies have shown correlation between rosetting and severe malaria (CM and severe anaemia) in Africa (Carlson et al., 1994; Heddini et al., 2001; Newbold et al., 1997; Rowe et al., 1995; Treutiger et al., 1992). However, the rosetting phenotype is not present in all *P. falciparum* isolates.

There are a number of RBC surface receptors and serum factors that have been shown to be involved in rosetting. Rosetting isolates have a preference for A or B blood groups, and form larger and stronger rosettes (Barragan et al., 2000 b; Carlson and Wahlgren, 1992; Rowe et al., 1995; Rowe et al., 2007; Udomsangpetch et al., 1989). The rosettes in blood group O are smaller and weaker, compared to rosettes formed in the A or B blood groups (Barragan et al., 2000 b). Blood group A has been associated with severe malaria (Pathirana et al., 2005; Rowe et al., 2007), while blood group O has shown protection against severe malaria disease due to reduction of rosettes (Loscertales et al., 2007; Rowe et al., 2007).

Complement receptor 1 (CR1) is a membrane glycoprotein found on the surface of RBC, monocytes, granulocytes, dendritic cells, B cells and T cells. CR1 is an immune-regulatory protein involved in the control of complement activation and immune clearance (Krych-Goldberg et al., 2002; Vik and Wong, 1993). The expression of CR1 on RBCs surface varies and is in the range of 50- 1 200 molecules per cell (Wilson et al., 1986). A study showed that CR1-deficient RBCs were unable to form rosettes with laboratory strains (Rowe et al., 1997). This was later confirmed with a monoclonal antibody against CR1 that showed to reverse rosettes in laboratory strains and clinical isolates from Africa (Rowe et al., 2000). CR1 gene polymorphisms have been reported to be highly frequent in populations living in endemic areas of Papua New Guinea, and have been linked to protection against severe malaria disease (Cockburn et al., 2004).

Heparan sulphate (HS) is found on the surface of RBC and is involved in rosetting. HS and other sulfated glycans inhibit or disrupt rosettes, at variable levels for different *P.falciparum* isolates and laboratory strains (Barragan et al., 2000 a; Carlson et al., 1992; Rowe et al., 1994). Modified heparin without anti-coagulant activity was shown to effectively inhibit rosettes in clinical isolates and laboratory strain (Vogt et al., 2006).

Serum proteins including non-immune IgM, non-immune IgG, von Willebrand factor and fibrinogen are involved in rosette formation in *P. falciparum* laboratory strains and clinical isolates (Flick et al., 2001; Scholander et al., 1998; Treutiger et al., 1999). Binding of non-immune immunoglobulin to the surface of pRBC in clinical isolates from children is common and associated with severe malaria disease (Heddini et al., 2001; Scholander et al., 1998). The role of IgM in rosetting has been thoroughly studied and IgM polymerization is essential for binding (Ghumra et al., 2008).

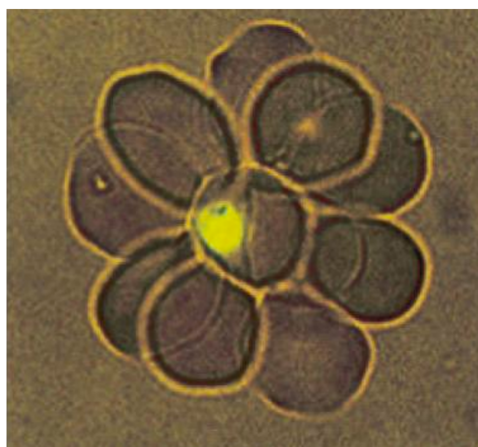

Figure 3. Rosetting trophozoite pRBC binding to several unparasitized RBC. (Adapted from (Heddini et al., 2001)).

1.5 *PLASMODIUM FALCIPARUM* MEROZOITE INVASION

Merozoite invasion of the RBC is a well-orchestrated high-specific multistep process (Figure 4), mediated by specific molecular interactions and signal transduction events between host receptors and parasite ligands. Invasion is executed with remarkable precision and retains the host cell integrity. Greater in depth knowledge understanding of this complex mechanism would aid the vaccine development. The micronemes and rhoptries located in the apical organelles play a crucial role in the host cell invasion (Cowman and Crabb, 2006). Parasite invasion occurs approximately 28 seconds after primary contact. The invasion is divided into two distinct dynamic “pre-invasion” and “classical invasion” stages. During the “pre-invasion”, the merozoite attach to the RBC and is involved in RBC deformation. This allows the merozoite to be enfolded at the point of attachment. This process takes 11 seconds and the RBC returns to its biconcave shape before second stage. The “classical invasion” involves the internalization of the merozoite within the RBC in 17 seconds. This is followed by echinocytosis, a deformation of the pRBC lasting for 10-15 minutes before the cell resumes normal shape (Dvorak et al., 1975). The rapid invasion event and the small size of merozoites (1.5 μm) contribute to the challenges to study the multi-step processes involved in the invasion. However, studies using light microscopy and electron microscopy have suggested that the merozoite invasion includes four main steps; I) egress of the merozoite, II) initial attachment, III) merozoite reorientation and irreversible tight junction formation and IV) merozoite entry (Aikawa et al., 1978; Cowman and Crabb, 2006; Dvorak et al., 1975; Gilson and Crabb, 2009).

1.5.1 EGRESS OF MEROZOITES

The initial step in merozoite invasion is the process known as egress. This process involves discharge from the parasitophorous vacuole (PV) and the host cell membrane of a mature schizont, containing 16 or more merozoites. Biochemical changes and intra-cellular pressure in the late stage mature pRBC cause a destabilization of the cytoskeleton, and results in an explosive rupture event releasing merozoites (Glushakova et al., 2005). Proteases are believed to be involved in egress, and this is a two step process including destruction and rupture of PVM followed by host cell membrane (Salmon et al., 2001). Proteases involved in the egress include cytoskeleton-degrading cysteine protease falcipain-2, aspartic protease plasmepsin II and serine repeat antigen (SERA) family localized in the PMV (Hanspal et al., 2002; Hodder et al., 2003; Miller et al., 2002b). The SERA protein family is also involved in

the sporozoite egress from oocysts (mosquito stage), indicating a role in the cell rupture (Aly and Matuschewski, 2005). Furthermore, a plant-like calcium dependent protein kinase, PfCDPK5, which is expressed in merozoites, is involved in egress (Dvorin et al., 2010b).

1.5.2 INITIAL ATTACHMENT

The primary contact between the merozoite and the RBC is reversible, and occurs possibly through glycosylphosphatidylinositol (GPI) anchored merozoite surface proteins (MSPs) and their associated partners. There are nine GPI anchored proteins found on the merozoite surface. These proteins share other similarities besides the GPI anchor, including cystein rich domains that could be significant in adherence. The most dominant and abundant GPI anchored MSP is the merozoite surface protein-1 (MSP-1), which is thought to be essential for parasite survival and is a major vaccine candidate (Holder and Freeman, 1982; Miller et al., 1993). MSP-1 has been considered to be essential for initiating the primary contact to the RBC. The initial contact is of low affinity and reversible, but important, and the parasite must differentiate the RBC's components for invasion (Bannister and Dluzewski, 1990). Initial attachment of the polar merozoites occurs at any point of the surface.

Peripheral proteins found in one of the broad protein families such as the MSP-3/MSP-6, the MSP-7 family, acidic basic repeat antigen (ABRA), glutamine rich protein (GLURP), Pf41 and the SERA protease family are candidates for binding to RBC receptors. These proteins are secreted into the PVM during the mature pRBC stage, and bind to the surface of developing merozoites, via interaction with a GPI anchored protein such as MSP-1 (Pachebat et al., 2001; Sanders et al., 2006). Besides the MSPS, there are a number of proteins stored in the apical organelles that bind specifically to the RBC receptor and are involved in the invasion. There is especially the *P.falciparum* apical membrane antigen-1 (PfAMA-1) that is highly conserved in the apicomplexan phylum. PfAMA-1 is crucial for establishing the interaction to initiate the invasion of *P.falciparum* and *T.gondii* (Mital et al., 2005; Triglia et al., 2000). Before invasion, the PfAMA-1 is translocated to the merozoite surface. PfAMA-1 has also been shown to be expressed in *P.falciparum* sporozoites and is believed to be essential for invasion of hepatocytes (Silvie et al., 2004). The secretion regulation of rhoptries and micronemes to the merozoite surface that allows receptor binding is an essential step in the invasion. Singh *et al.* demonstrated in a recent study that released merozoites experience a rise in cytoplasmic calcium as response to a rise in potassium ion concentration and exposure to the extracellular milieu of the blood. Subsequently, this process triggers sequential secretion of merozoite antigens, such as PfEBA175 and PfAMA-1, to the merozoite surface and initiates binding. The receptor and ligand interaction restores cytoplasmic calcium levels and releases the rhoptry content. Blockage or inhibition of this signal pathway involved in apical organelle discharge could be a target for drug development (Singh et al., 2010).

1.5.3 MEROZOITE REORIENTATION AND IRREVERSIBLE TIGHT JUNCTION FORMATION

The initial contact results in RBC deformation, and this allows reorientation of the merozoite so that the apical end points towards the RBC membrane. There is an irreversible tight-junction formation between the merozoite and the RBC membrane. The tight junction is a ring of close contact. After the tight junction formation there is activation of the invasion process involving a release of proteins from the mircronemes and rhoptries. Activation of the

invasion process involves a release of proteins from the mircronemes and rhoptries. There are two main protein families; the erythrocytic binding like (EBL) family and the reticulocyte binding homolog (PfRh or PfRBL) family (Duraisingh et al., 2003b; Miller et al., 2002a; Stubbs et al., 2005; Triglia et al., 2005), which are prime candidates for tight junction formation. The EBL family are found in the micronemes and include six erythrocyte binding antigens (EBAs) in the *P. falciparum*; PfEBA-175, PfEBA-140 (or BAEBL), PfEBA-181 (JSEBL), PfEBL-1, PfEBA-165 (PEBL) and MAEBL (Blair et al., 2002; Camus and Hadley, 1985; Maier et al., 2003; Peterson and Wellems, 2000; Triglia et al., 2001). The PfRh proteins were identified as homologs of rhoptry proteins in *P . yoelii* and *P . vivax (Galinski et al., 1992; Preiser et al., 2002)*. *P. falciparum* express five members of PfRh; PfRh1 (PfRBP1), PfRh2a (PfRBP2a), PfRh2b (PfRBP2b), PfRh4 (PfRBP4), and PfRh5 (PfRBP5), and a sixth PfRh3 that is not expressed as a protein (Duraisingh et al., 2003b; Stubbs et al., 2005; Triglia et al., 2005). Both protein families are believed to be essential for merozoite invasion (Duraisingh et al., 2003a; Duraisingh et al., 2003b; Lopaticki et al., 2011; Maier et al., 2003; Stubbs et al., 2005; Triglia et al., 2005), as well as PfAMA1. During merozoite invasion, PfAMA-1 has multiple roles; apical reorientation (Mitchell et al., 2004), erythrocyte binding (Fraser et al., 2001; Kato et al., 2005), attachment and rhoptry secretion (Mital et al., 2005), formation of the moving junction and intracellular replication (Collins et al., 2009; Treeck et al., 2009). PfAMA-1 is believed to be essential for the apical interaction through adhesins located at the neck of the rhoptries and in the micronemes. It is thought to form an important link between the weak link of MSPs and the irreversible tight formation with micronemal proteins (Mitchell et al., 2004). Recent studies have shown that the interaction between PfAMA1 and rhoptry neck protein 2 (RON2) is critical for tight junction formation with RBC, and is an irreversible step that allows parasite invasion (Srinivasan et al., 2011). Specific antibodies against PfAMA-1 have been shown not to prevent initial attachment, but to block the reorientation of the merozoite (Mitchell et al., 2004).

After apical attachment there may be a discharge of mediators into the RBC by the merozoite, but these have not been clearly identified. Electron microscopy of the merozoite invasion has suggested that there is a transfer of material (Aikawa and Miller, 1983).

1.5.4 MEROZOITE ENTRY

During the merozoite entry, the tight junction moves from the apical to the posterior end of the merozoite in multiple steps of events driven by the actin-myosin motor. The motor complex has been studied in *T . gondii*, and this seems to be conserved in the apicomplexa, including *Plasmodium spp*. The thrombospondin-related anonymous protein (TRAP) is thought to link extracellular adhesins to the parasite cytoskeleton (Kappes et al., 1999; Sultan et al., 1997). Both TRAP and circumsporozoite protein (CSP) are believed to be involved in the sporozoite invasion of hepatocytes. A study suggests that the cytoplasmic tail of TRAP in *P . falciparum* links to the actin-myosin motor through aldolase (Buscaglia et al., 2007). Merozoite TRAP (MTRAP) is thought to provide a similar function for merozoite invasion in RBC (Baum et al., 2005). As the tight junction is moving towards the posterior end, proteins involved in the invasion process need to be removed. This process is known as the “shedding” or removal of the fuzzy coat covering the merozoite surface, and involves a serine protease (subtilase) called SUB2 localized in the micronemes (Harris et al., 2005). Once the merozoite has entered the RBC the PV encloses it. When the merozoite has been internalized in the RBC it undergoes echinocytosis, due to water loss stimulated by an efflux of potassium and chloride ions. The

calcium efflux could also be due to the formation of PV and PVM around the invading merozoite (Desai et al., 1993; Lew and Tiffert, 2007). The PVM is formed from invagination of the RBC membrane during invasion. The PVM fuses and separates a biochemical and physical barrier between the host cytosol and the parasite. The parasites remain enclosed within the PVM during the intra-erythrocytic development phase (Aikawa et al., 1979).

Dense granules are believed to be discharged after complete parasite entry, and are thought to be involved in host cell modifications.

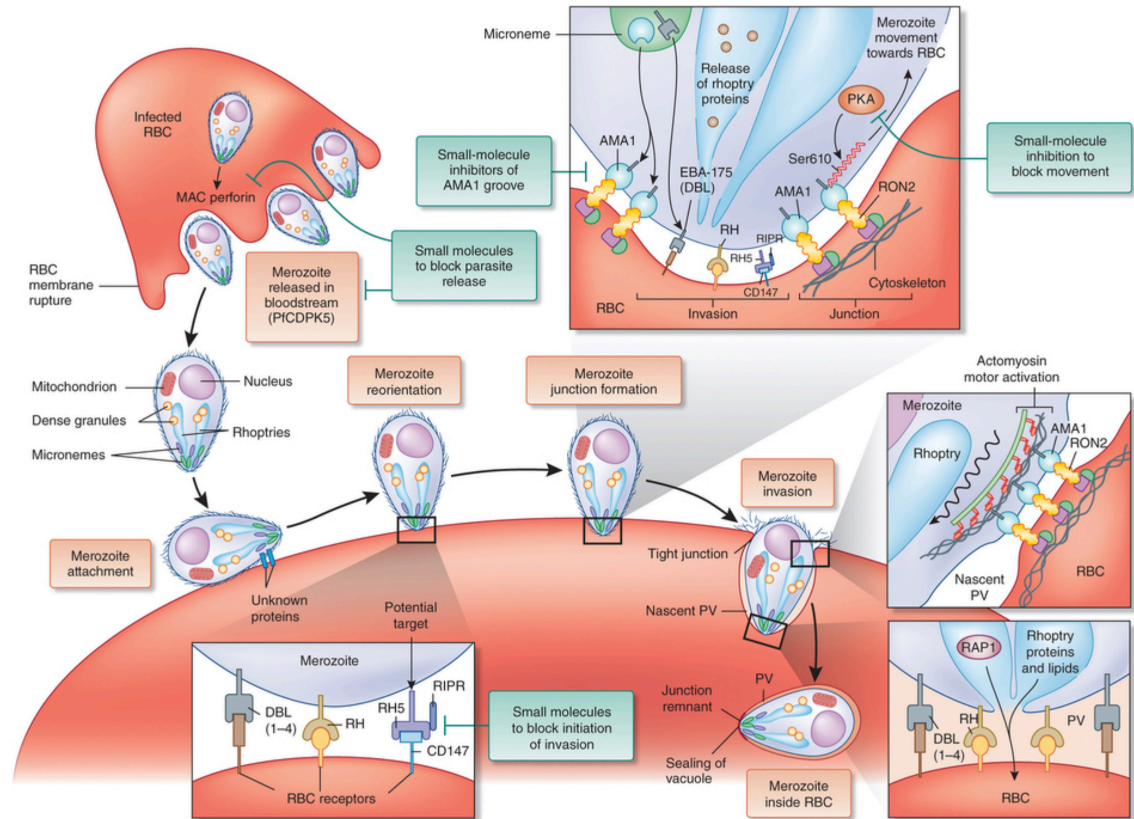

Figure 4. *Plasmodium falciparum* merozoite invasion into red blood cells (Adapted from (Miller et al., 2013) and published with permission from Nature Publishing Group).

1.6 SIALIC ACID PATHWAYS

Invasion phenotypes utilized by parasites can be divided into two main pathways I) sialic acid (SA)-dependent invasion, demonstrated by poor invasion of neuraminidase treated RBC (neuraminidase cleaves SA on the RBC surface), and II) SA-independent invasion, demonstrated by efficient invasion of neuraminidase treated RBC (neuraminidase sensitive) (Camus and Hadley, 1985; Duraisingh et al., 2003b; Maier et al., 2003; Reed et al., 2000; Sim et al., 1994).

Besides the fact that both PfRh and PfEBA proteins are able to employ alternative invasion pathways, it is believed that each parasite ligand binds to a specific receptor (Figure 5). There must be reasons for why the parasites maintain a large number of genes and mechanisms to regulate the expression. Firstly, the RBC surface is highly diverse among individuals, and each individual RBC formula can vary by age and sex, and in response to infection and nutrients. Secondly, in endemic areas most individuals have acquired some kind of mechanism to avoid binding to specific parasite ligands. Finally, flexible invasion pathways increase chances for parasite survival, and optimize the ability to establish infection in an individual.

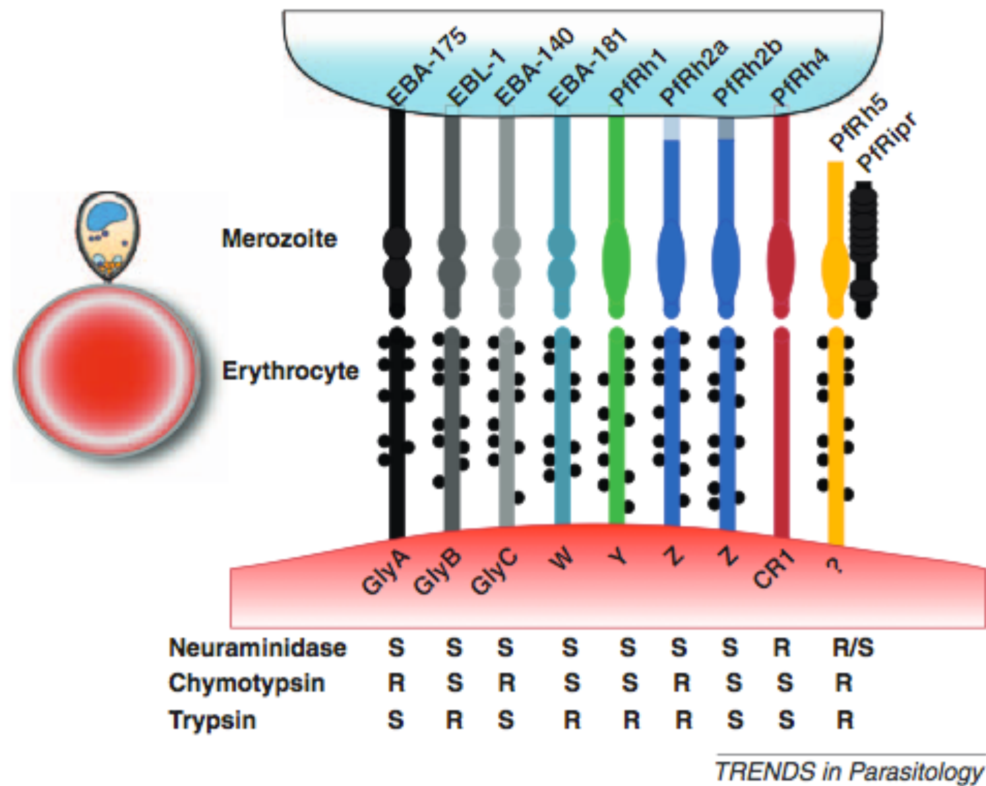

Figure 5. Host cell receptors on the RBC shown to bind specific *P.falciparum* ligands (Adapted from (Tham et al., 2012) and published with permission from Trends in Parasitology). **NOTE**: Receptor for PfRh5 was recently identified as Basigin ((Crosnier et al., 2011)).

1.7 *PLASMODIUM FALCIPARUM* MEROZOITE ANTIGENS

To date a number of merozoite antigens have undergone extensive studies as potential candidates for vaccine development. A panel of merozoite antigens were selected and included in the studies discussed in this thesis. In the following section the apical membrane antigen 1 (AMA-1), the merozoite surface proteins (MSP1, MSP2), erythrocyte binding like proteins (PfEBA-175, PfEBA-140, PfEBA-181) and finally reticulocyte binding ligand proteins (PfRh2a, PfRh2b, PfRh4) will be discussed.

1.7.1 APICAL MEMBRANE ANTIGEN 1 (AMA-1)

P . falciparum apical membrane antigen 1 (PfAMA-1) (Peterson MG, 1989) is one of the most extensively studied and best characterized invasion ligands. It was initially discovered 30 years ago in *P . knowlesi* (Deans et al., 1988). Additionally, PfAMA-1 was the first rhoptry gene to be sequenced and is also a promising vaccine candidate (Dutta et al., 2002; Kocken et al., 2002; Peterson MG, 1989).

PfAMA-1 is expressed during *P . falciparum* schizogony, and is synthesized to an 83 kDa precursor that is proteolytically processed in the micronemes to yield a 66 kDa surface protein (Narum and Thomas, 1994). The mature 66 kDa form of PfAMA-1 is found in the micronemes (Healer et al., 2002), and it is proteolytically processed into smaller fragments (44-48 kDa molecules) during translocation to the merozoite surface before invasion. PfAMA-1 is a type 1 integral membrane protein with large extracellular domain and a short cytoplasmic tail.

The ectodomain of AMA-1 is mainly composed of three domains; the N-terminal domain I, the central domain II and the C-terminal domain III (Nair et al., 2002; Pizarro et al., 2005). The PfAMA-1 sequence is highly conserved among the *Plasmodium spp*. (Cheng and Saul, 1994; Dutta et al., 1995; Marshall et al., 1989; Waters et al., 1990). Even though PfAMA-1 lacks the low-complexity regions found in other merozoite proteins, a relative large number of point mutations are found in this gene. Population genetic analyses have shown that these polymorphisms are especially found in domains I and III (Cortes et al., 2003; Polley and Conway, 2001) and are under balancing selection, and are targets of protective immune response which makes them immunologically significant. Studies have shown that conserved and strain-specific epitopes of PfAMA1 are targets of invasion inhibitory antibodies (Cortes et al., 2005; Healer et al., 2004; Kocken et al., 2002; Narum et al., 2006). Multi-allele immunization showed an increase of cross-reactive antibodies against PfAMA1(Kusi et al., 2009).

1.7.2 MEROZOITE SURFACE PROTEIN 1

The merozoite surface protein 1 (MSP1) was described in the 1980s, and is well-studied merozoite antigen. Early vaccine studies with purified MSP1 showed protection against *P . yoelii* infection in a rodent model (Holder and Freeman, 1981). MSP1 is believed to be involved in initial contact with the RBC binding via the surface receptor Band 3 and also heparin-like-molecules (Boyle et al., 2010; Goel et al., 2003; Li et al., 2004). MSP1 is the most abundant surface protein and constitute approximately 40% of all GPI-anchored MSPs. MSP1 is synthesized during schizongony to a 195 kDa precursor, and undergoes multiple proteolytic cleavages (Holder, 1988; Holder et al., 1992). At the time of merozoite egress, MSP1 is primarily processed by a subtilisin-like protease, SUB1, to produce four polypeptide fragments; an 83 kDa N-terminal fragment (MSP1-83), two internal fragments of 30 and 38 kDa (MSP1-30 and MSP1-38) and a C-terminal 42 kDa fragment (MSP1-42) with an anchor (Pachebat et al., 2001; Stafford et al., 1996). The MSP1 complex on the merozoite surface undergoes a secondary process with shedding during invasion. SUB2 cleaves MSP1-42 into 2 fragments; 33 kDa (MSP1-33) and 19 kDa (MSP1-19). The MSP1 complex and MSP1-33 are shed from the surface in a soluble form, and the C-terminal of MSP1-19 is membrane bound via GPI anchor (10% of the protein) and enters the newly invaded RBC (Blackman et al., 1990; Harris et al., 2005). MSP1-19 has two epidermal growth factor (EGF)-like domains, and after the invasion they are transferred to the forming food vacuole and remain during the intracellular parasite development (Dluzewski et al., 2008).

The MSP1 gene has 17 variable and conserved blocks separated by semi-conserved or conserved regions (Tanabe et al., 1987). The N-terminal region of MSP-1, known as block 2, is a highly polymorphic region with numerous known variant sequences from globally various parasite isolates (Jiang et al., 2000; Miller et al., 1993). The block 2 sequence can be divided into four major allelic types (K1, MAD20, RO33 and MR) (Roy et al., 2008; Takala et al., 2002). The sequence of MSP1-19 is highly conserved with six non-synonymous single nucleotide polymorphisms (SNPs) at amino acid positions. New genetic variants can be derived from the single nucleotide mutations maintained by positive natural selection (Ferreira et al., 2003; Miller et al., 1993).

1.7.3 MEROZOITE SURFACE PROTEIN 2

Merozoite surface protein 2 (MSP2) is another potential vaccine candidate (Anders et al., 2010). MSP2 is a ~25 kDa highly abundant GPI-anchored membrane protein. It was initially identified using a monoclonal antibody in *P. falciparum*, and a couple of years later the gene was identified in an expression library (Anders et al., 2010; Smythe et al., 1988; Stanley et al., 1985). Sequence polymorphisms in MSP2 genes have described in laboratory strains and field isolates (Anders et al., 2010; Felger et al., 1994; Fenton et al., 1991; Prescott et al., 1994; Smythe et al., 1988; Stanley et al., 1985). The MSP2 gene is one of the most polymorphic of all merozoite protein genes and consists of 5 blocks (Snewin et al., 1991). The MSP2 gene includes a highly conserved 5´and 3´sequence (N- and C-terminally) flanked by a central variable region. The variable region has sequence repeats flanked by non-repetitive dimorphic sequences, and these form two distinct MSP2 alleles, grouped into the 3D7 and FC27 allelic families (Felger et al., 1994; Fenton et al., 1991; Smythe et al., 1990). The polymorphism has probably arisen from the selection pressure of protective immune responses (Barry et al., 2009; Weedall and Conway, 2010). The variable central repeats define the individual alleles of MSP2 and point mutations have been found in both allele families (Felger et al., 1994). Furthermore, MSP2 is commonly used as single marker for molecular characterization of field malaria parasites. PCR typing of MSP2 gene allows the determination of malaria infection indicators such as diversity of *P.falciparum* strains and multiplicity of infection (MOI), which may give a description of the malaria situation in a given study location (Kiwuwa et al., 2013; Ntoumi et al., 1995) and MSP2 assessment is equally reliable to MSP1 in field diagnosis of malaria (Smith et al., 1999).

1.7.4 ERYTHROCTYE BINDING LIKE (EBL) PROTEIN FAMILY

The EBL protein family is thought to play a crucial role in the RBC recognition, tight junction formation and eventually in the invasion. *P. falciparum* can invade Duffy negative and positive RBC, and can use a number of distinct receptors (Dolan et al., 1990; Miller et al., 1977; Mitchell et al., 1986). The EBLs are located in the sub-telomeric region of different chromosomes, and encoding genes are associated with parasite virulence and pathology. The proteins have conserved exon-intron structures, splicing boundaries and contain two extracellular regions that have conserved cysteine and hydrophobic amino acid residues (Adams et al., 1992; Gardner et al., 2002).

The EBA proteins found in *P. falciparum* bind to sialic acid dependent receptors, and this is known as sialic acid dependent pathway, which is important for mediation of alternative invasion pathways (Pasvol, 2003).

1.7.4.1 ERYTHROCYTE BINDING ANTIGEN 175

P . falciparum erythrocyte binding antigen 175 (PfEBA-175) consists of a 175 kDa protein and is localized in the micronemes (Camus and Hadley, 1985; Orlandi et al., 1990). The single copy *eba-175* gene is found on chromosome 7, and is constituted of four exons and seven regions, I to VII. The region II is found in the central part of the gene, a highly dimorphic segment in this region, and is conserved among strains of *P. falciaprum*. The region III-V (RIII-V) of PfEBA-175 is dimorphic, and the parasites strains encode either a C (Camp strain) or F (FCR3 strain) allelic haplotype (Sim et al., 1990; Toure et al., 2006; Toure et al., 2001).

The RBC receptor for PfEBA-175 is glycophorin A (Gly A), which is present as a dimer on the RBC surface. It seems like the majority of cultured parasites use the PfEBA-175/GlyA pathway, and a minority use alternative ligand-receptor combinations. Enzyme treatment of RBC with neuraminidase (which removes sialic acid) or trypsin (removes other proteins) did not completely inhibit the invasion but forced the parasite to use other receptors for invasion in one study (Dolan et al., 1990; Gaur et al., 2003; Sim et al., 1994). At first the parasite replication was dramatically reduced on initial exposure to enzyme treated RBC, but the parasites adapted with time and invaded with the same efficiency as for untreated RBC. This is strong evidence that *P. falciparum* can adapt and switch to using a new receptor on the RBC, resulting in using different invasion pathways. Interestingly, once the parasites are removed from treated RBC and cultured in untreated RBC it reverts back to the PfEBA175/GlyA pathway. These findings have been confirmed with PfEBA-175 knock out parasites, that were unable to invade through the GlyA receptor (Duraisingh et al., 2003b; Reed et al., 2000). However, the parasites have the ability to switch invasion pathways. Stubbs *et al.* found that gene disruption of PfEBA175 was associated with upregulation of PfRh4 and the loss of using the sialic acid dependent pathway (Stubbs et al., 2005). The PfEBA175/GlyA interaction could be a potential target to consider for vaccine-induced antibodies.

1.7.4.2 ERYTHROCYTE BINDING ANTIGEN 140

P. falciparum erythrocyte binding antigen 140 (PfEBA-140) is a 140-kDa protein. The *eba140* gene has identical intron/exon structure as that described for PfEBA-175. PfEBA-140 share 30% similarity to the EBA175 protein sequence, and PfEBA-181 has 37% sequence similarity to PfEBA-140 (Adams et al., 1992; Gilberger et al., 2003; Maier et al., 2003). GlyC on the RBC has been identified as receptor for PfEBA-140 (Maier et al., 2003; Mayer et al., 2002). The binding profile of PfEBA-140 receptor is trypsin and neuraminidase sensitive, but chymotrypsin resistant. Soluble sialic acid was not capable of inhibiting PfEBA-140 RBC binding. These findings demonstrate that receptor glycans containing sialic acids are crucial for receptor recognition, and that the backbone of GlyC plays a role in the binding (Jiang et al., 2000; Mayer et al., 2006). Functional studies of PfEBA-140 knock out parasites showed binding to GlyA and GlyB, although the binding was dramatically reduced compared to its GlyC binding (Maier et al., 2003). To date, only four polymorphic mutants have been identified in RII PfEBA-140, and they are found in the F1 domain. The polymorphisms in PfEBA-140 changes the binding profile of the ligand to enzyme treated RBC. The change in binding profile suggests that the PfEBA-140 has an ability to interact with other receptors, and to mediate alternative invasion pathways independent from GlyC. Additionally, polymorphisms has shown to diminish the affinity of PfEBA-140 for RBC (Maier et al., 2009a; Mayer et al., 2002). Recent studies suggest that the PfEBA-140 binds to glycosaminoglycans (GAGs) on the RBC and may have a functional role during PfEBA-140-mediated invasion (Boyle et al., 2010).

In malaria endemic regions of Papua New Guinea there is a high frequency of Gerbich negativity. This phenotype is due to loss of exon 3 within the GlyC gene. This prevents the PfEBA-140 RBC binding and inhibits the invasion. This phenotype is present in the Melanesian population and has probably arisen through natural selection by severe malaria (Maier et al., 2003).

1.7.4.3 ERYTHROCYTE BINDING ANTIGEN 181

P. falciparum erythrocyte binding antigen 181 (PfEBA-181) is encoded by a 181 kDa protein and it is expressed in merozoites and schizonts. Within the mirconemes, PfEBA-181 is co-localized with PfEBA-175 at the apical end of free merozoites. Moreover, both PfEBAs are co-localized in the mature schizont (Gilberger et al., 2003). PfEBA-181 share sequence similarity with PfEBA-175 and PfEBA-140 (especially the F1/F2 domain), 25.3% and 24.6% respectively. Eight polymorphisms have been identified in the RBC binding domain of PfEBA-181. However, there are fewer mutations found in PfEBA-181 and PfEBA-140 compared to PfEBA-175 (Gilberger et al., 2003; Mayer et al., 2004). The point mutations in PfEBA-181 lead to recognition of different receptors on the RBC. There is a possibility that the polymorphisms in the RBC binding domain of PfEBA-181 give the parasite population a survival advantage in a genetically diverse human population.

The exact receptor for PfEBA-181 is unknown. Previous characterization of PfEBA-181 showed binding to the RBC (sialic acid dependent manner) via a trypsin resistant, chymotrypsin sensitive and neuraminidase sensitive receptor. Moreover, disruption of the *eba-181* gene did not have any effect on the parasite invasion (Gilberger et al., 2003; Mayer et al., 2004). PfEBA-181 has been shown to bind a highly conserved 10 kDa domain in 4.1R found on the RBC membrane. This interaction could have multiple functions during parasite invasion, however the significance of this *in vivo* is unknown (Lanzillotti and Coetzer, 2004; Lauterbach et al., 2003).

1.7.5 RETICULOCYTE BINDING PROTEIN HOMOLOGUE (Rh) FAMILY

The second family involved in merozoite invasion is the reticulocyte binding-like (RBL) proteins. These include PfRh1, PfRh2, PfRh4, and PfRh5 and are large proteins with a C-terminal trans-membrane domain. All PfRhs except PfRh4 share a homologous region of around 500 amino acids. There are some conserved parts shared between *P. falciparum* and *P. vivax* RBL proteins (Rayner et al., 2000). The expression patterns in the RBL proteins and apical location are consistent with the role during the invasion process. The PfRh proteins invade mainly through the sialic acid independent pathway (except for PfRh1).

1.7.5.1 RETICULOCYTE BINDING PROTEIN HOMOLOGUE 2 (Rh2)

There are two *P. falciparum* reticulocyte protein homologues 2, (PfRh2a and PfRh2b), and they are encoded by two genes that are adjacent to each other on chromosome 13, and are thought to have arisen by gene duplication. These genes are around 9 kb in length and are highly homologous in sequence (80%) and share more than 8 kb of nucleotide sequence at the 5´end before a divergent and unique 3´end (Rayner et al., 2000). However, the difference in the gene sequence is at the C-terminal domain, which determines the phenotype (Dvorin et al., 2010a). The proteins are large hydrophilic proteins and they are predicted to be more than 350 kDa, with a N-terminal signal sequence and a single trans-membrane domain near the C-terminal end (Duraisingh et al., 2003b; Rayner et al., 2000). The proteins are found at the apical end of the merozoite, more closely in the neck of the rhoptries (Cowman and Crabb, 2006), and they are located at the junction during invasion. It has been proposed that they have an important adhesive function during invasion. Most strains have the PfRh2a and PfRh2b genes, but not all parasites express the proteins (Duraisingh et al., 2003b; Taylor et al., 2002). Both

PfRh2a and PfRh2b have been knocked out in the parasite 3D7 (Duraisingh et al., 2003b). PfRh2b disrupted parasites showed a significantly altered invasion in enzyme treated RBC compared to the wild type clone. PfRh2b have been implicated to have an important role in invasion by mediating alternative pathways, and trypsin resistant receptor Z has been shown as essential for the binding to the RBC (Cowman and Crabb, 2006). On the other hand, knock-out of PfRh2a did not show any difference in invasion phenotype compared to the wild type 3D7 parasite (Duraisingh et al., 2003b). Moreover, disruption of PfRh2a and PfRh2b has also in other studies been shown to be important for sialic acid independent invasion (DeSimone et al., 2009).

1.7.5.2 RETICULOCYTE BINDING PROTEIN HOMOLOGUE 4 (Rh4)

P. falciparum reticulocyte binding protein homologue 4 (PfRh4) is found on chromosome 4. PfRh4 is a 220 kDa type I trans-membrane protein and is present in the rhoptries, and like all the other proteins in the PfRh family it undergoes processing to a 160 kDa protein (Cowman and Crabb, 2006; Gaur and Chitnis, 2011; Tham et al., 2012). Complement receptor 1 (CR1) was identified as a receptor for the sialic acid independent invasion pathway in numerous laboratory strains and field isolates (Spadafora et al., 2010). At the same time several studies showed that PfRh4 was essential for the sialic acid independent pathway. It was shown that PfRh4 binding strongly correlated with the CR1 level on the RBC surface. Invasion through sialic acid-independent pathways was reduced in low CR1 expressing RBC. Additionally, soluble CR1 competitively blocked binding of PfRh4 to the RBC surface, and hence inhibited sialic acid independent invasion (Tham et al., 2009). Activation of PfRh4 expression allowed the parasite to switch usage of receptors from sialic acid dependent to sialic acid independent pathways, and this provides alternative invasion pathways utilized by the parasite (Stubbs et al., 2005).

Expression of the PfRh4 protein varies among parasite isolates but has been found in both field isolates from Africa (Gomez-Escobar et al., 2010; Nery et al., 2006) and laboratory-adapted isolates (Gaur et al., 2006; Stubbs et al., 2005; Tham et al., 2011).

1.8 NATURALLY ACQUIRED IMMUNITY TO MALARIA

Acquired immunity can either be active or passive. Active acquired immunity is obtained from development of antibodies in response to an antigen, either from vaccination or infectious diseases. Passive acquired immunity is developed by the prenatal or postnatal transfer of protective immunoglobulin from mother to child or by injection of antiserum.

Naturally acquired immunity to malaria is not sterile, and individuals living in endemic countries only acquire protective immunity after repetitively exposure to the parasite (Figure 6) (Bull et al., 1999; Trape et al., 1994). There is high *P. falciparum* malaria associated morbidity and mortality among infants and young children, but older children and adults have circulating parasites, with or without clinical disease. Studies have shown that immunity to severe malaria is the first in line immunity that develops in children in malaria endemic countries (Bull et al., 1999; Gupta et al., 1999a; Trape et al., 1994). Naturally acquired immunity is comprised in pregnant women, and adults that move from endemic countries loose their acquired immunity temporarily. Women become resistant to PAM after repeated pregnancies and infection (Fried et al., 1998). Severe malaria in newborn infants born to mothers in endemic areas is rare in the

first months, most likely because of the placental transfer of immunoglobulin G (IgG) (Snow et al., 1998).

Individuals living in high malaria transmission areas reach immunity faster compared to individuals in low transmission areas. During the 1980s malaria interventions reduced malaria exposure in Madagascar, and natural acquired immunity was lost. This lead to a catastrophic event with a malaria endemic killing more than 40 000 people (Romi et al., 2002). The recurrent exposure to hyper- and holo-endemic malaria protects the majority of the individuals and kills only a minority. Malaria immunity is dependent on age, transmission intensity and exposure to parasite.

Protective immunity to malaria is multi-faceted and includes immunological responses to both pre-erythrocytic and erythrocytic stages (Langhorne et al., 2008; Marsh and Kinyanjui, 2006). Studies have demonstrated that passive transfer of IgG from clinically immune donors to individuals infected with *P.falciparum* resulted in reduced parasitemia and clearance of clinical symptoms (Bouharoun-Tayoun et al., 1990; Cohen et al., 1961; McGregor et al., 1963).

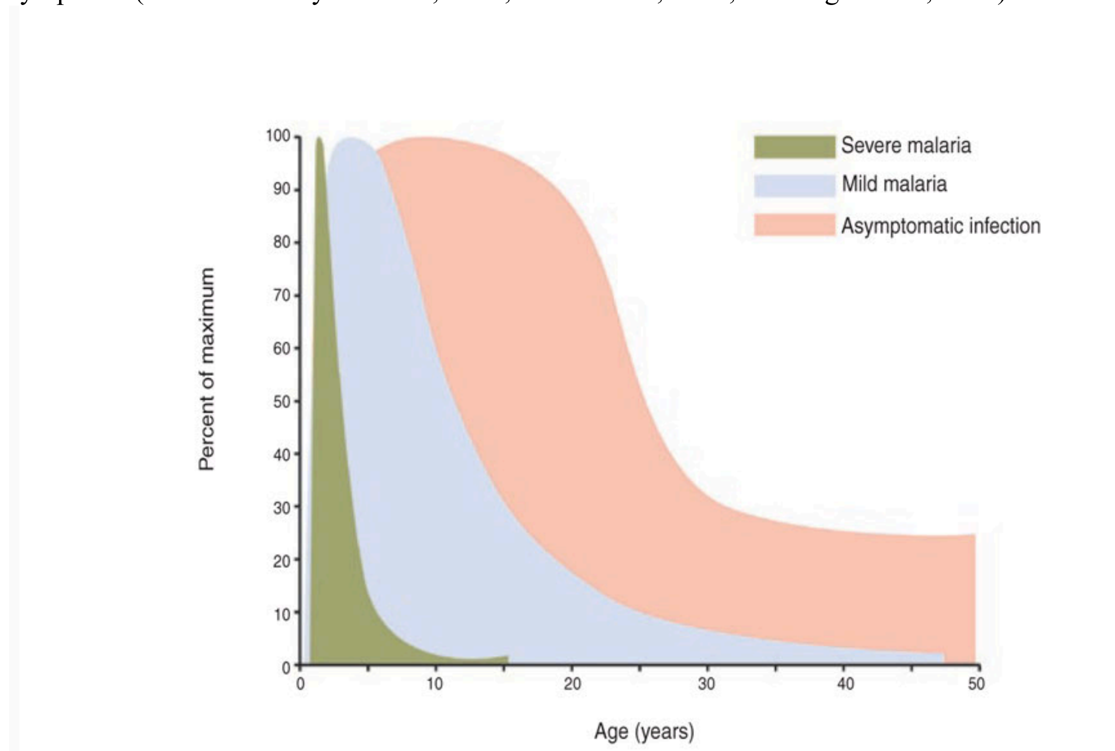

Figure 6. Acquisition of immunity to malaria in endemic areas (Adapted from (Langhorne et al., 2008) and reproduced with permission from Nature Publishing Group).

1.8.1 IMMUNOGLOBULIN M (IgM), IgG AND SUBCLASS IgG1-4 IN MALARIA

In malaria, there are a wide range of antibodies of different isotypes that are capable of inducing protective effects against malaria. In the following section, IgG and IgM will be briefly discussed.

1.8.1.1 IMMUNOGLOBULIN M (IgM)

Immunoglobulin M (IgM) is part of the first line host defense (Leoratti et al., 2008). The pentamer structure of IgM equips the molecule with functions such as multivalent binding to both antigens and receptors (Ye et al., 2010). Natural IgM has been shown to bind to the surface of pRBC via the Fc-part of the molecule, and is correlated with severe malaria in both laboratory strains and clinical isolates (Ghumra et al., 2008; Rowe et al., 2002b).

Some *P. falciparum* strains bind natural IgM, and this is found in parasites with specific virulence phenotypes such as rosetting (Rowe et al., 2002b), and CSA binding has been linked to placental malaria infection (Creasey et al., 2003; Fried et al., 1998). Studies suggested that elevated IgM levels are important for agglutination (Doolan et al., 2009), and for neutralization of pathogens (Czajkowsky et al., 2010). IgM is also a potent complement activator (Czajkowsky and Shao, 2009).

1.8.1.2 IMMUNOGLOBULIN G (IgG)

Immunoglobulin G (IgG) is the most abundant isotype found in humans. There are four IgG subclasses, IgG1-IgG4, named in order of their abundance in serum. The subclasses differ both in structure and in immunological functions (Nimmerjahn and Ravetch, 2008). IgG and IgG subclass responses have been reported in malaria. IgG1 and IgG3 are known as cytophilic antibodies and have been suggested to be the predominant antibody responses in *P . falciparum* infections (Bouharoun-Tayoun and Druilhe, 1992; Dobano et al., 2012; Roussilhon et al., 2007; Sarthou et al., 1997). Cytophilic IgG1 or IgG3 have been reported to be associated with lower parasitemia, lower risk for malaria attacks and with clinical protection from disease (Shi et al., 1996; Taylor et al., 1998).

IgG2 and IgG4 are non-cytophilic, and are speculated to be non-protective. However, elevated levels of IgG2 have been shown to be correlated with decreased risk of malaria infection, and more specifically in individuals that carry an allelic variant of FcγRIIA, which binds to IgG2 (Aucan et al., 2000; Garraud et al., 2003; Nasr et al., 2009). IgG4 is believed to compete with the binding of IgG1 and IgG3, and might be involved in blocking the cytophilic activity (Aucan et al., 2000; Garraud et al., 2003).

In malaria, the dynamics of subclass responses and their association with protection is probably important, but more studies are required to fully understand the immune responses against specific antigens and this knowledge could add in vaccine development.

1.8.2 ANTIBODY RESPONSES TO THE *P. FALCIPARUM* MEROZOITE ANTIGENS

Antibodies against *P. falciparum* are directed against different stages of the parasite during its life cycle. The function of antibodies in malaria infection and disease are the following; I) inhibition of merozoite invasion/intracellular growth, II) antibody-dependent

cellular killing with opsonization, and III) clearance of pRBC by antibody binding to the host cell surface, and inhibition of sequestration in the microvasculature (Blackman et al., 1990; Bouharoun-Tayoun et al., 1995).

It has been demonstrated that antibodies against several *P. falciparum* merozoite antigens are associated with protective immunity in longitudinal studies (Branch et al., 1998; Egan et al., 1996; Fowkes et al., 2010; John et al., 2005). Previous studies have reported associations between high levels of antibodies against several merozoite antigens and protection against symptomatic malaria, while others considered it more as marker of malaria exposure (Gupta et al., 1999b; Metzger et al., 2003; Polley et al., 2004; Richards et al., 2010). Several studies described invasion/growth inhibitory activities in sera from individuals living in malaria endemic areas (Bouharoun-Tayoun et al., 1990; Cohen et al., 1969; Dent et al., 2008). It has been reported that there are significant associations between growth inhibitory antibodies and reduced risk of clinical malaria (Crompton et al., 2010; Dent et al., 2008; John et al., 2004), while other findings were inconclusive (Corran et al., 2004; Perraut et al., 2005).
The following sections will discuss previous studies on antibody responses to the *P. falciparum* merozoite antigens included in this thesis work.

1.8.2.1 ANTIBODIES AGAINST PfAMA1

Individuals living in malaria endemic areas can have increased levels of antibodies against PfAMA1, which are strong inhibitors of parasite invasion (Bouharoun-Tayoun et al., 1990; Courtin et al., 2009; Nair et al., 2002). Few studies have directly measured inhibitory antibodies in relation to protection from malaria. Those conducted reported presence of antibodies against PfAMA1 associated with a reduced incidence of malaria (Greenhouse et al., 2011; Keh et al., 2012; Polley et al., 2004).

There is limited knowledge on acquired antibodies against the polymorphic and conserved epitopes of PfAMA1 (Polley et al., 2004). A recent study showed that antibodies that target the functional and polymorphic epitopes of PfAMA1, are acquired with altering exposure to malaria, and indicates some association with decreased risk of clinical malaria. These findings suggest that PfAMA1 could target of naturally acquired antibody response (Mugyenyi et al., 2013).

1.8.2.2 ANTIBODIES AGAINST MSP1-19

Several mechanisms have been suggested for how MSP1-19 antibodies mediate protection against malaria, including antibody binding and inhibition of MSP1-19 function and merozoite invasion, disruption of secondary processing of the protein, agglutination of merozoites, and opsonization of parasites by phagocytic cells (Blackman et al., 1990; Blackman and Holder, 1992; Gilson et al., 2008; McIntosh et al., 2007). It has been reported that antibodies against MSP1-19 are associated with control of malaria or protection from symptomatic *P. falciparum* malaria (Branch et al., 1998; Egan et al., 1996; Perraut et al., 2005; Riley et al., 1992), and was found in both infants and young children indicating that protection associated with MSP1-19 develops early (Branch et al., 1998; Shi et al., 1996). A recent study of IgG against MSP1-19 reported lack of association between growth inhibitory activity and protection from malaria or re-infection. This study suggests that antibodies against MSP1-19 might be marker of protective immunity and not directly involved in the protective mechanisms such as growth inhibition (Wilson et al., 2011).

1.8.2.3 ANTIBODIES AGAINST MSP2

Early studies showed that MSP2 specific monoclonal antibodies could inhibit parasite growth *in vitro* (Clark 98 MBP; Epping 88 MBP). Since then several studies have reported that antibodies to MSP2 might be involved in protection against malaria. A study in Papua New Guinea showed that antibodies against MSP2-3D7, but not against MSP2-Fc27, were associated with decreased incidence of clinical malaria (al-Yaman et al., 1995). Two studies in The Gambia reported significant association of IgG3 antibodies against MSP2 with reduced morbidity (Metzger et al., 2003; Taylor et al., 1998). A more recent study demonstrated that antibodies against MSP2 bound to the merozoite surface but did not inhibit invasion and instead got carried into the pRBC during invasion, and remained there up to 20 hours post infection without inhibiting intra-erythrocytic development (Boyle et al., 2013). These findings indicate that MSP2 specific antibodies might not be directly involved in invasion inhibition, but could still be involved in opsonization of merozoites and antibody-dependent cellular inhibition mediated by monocytes, and these are considered as important mechanisms in acquired immunity (Bouharoun-Tayoun et al., 1990; Flueck et al., 2009).

1.8.2.4 ANTIBODIES AGAINST PfEBAs

Antibody responses against PfEBA175 have been associated with age in endemic areas (Noland et al., 2008; Okenu et al., 2000; Persson et al., 2008; Richards et al., 2010). Studies in The Gambia observed that increasing antibody levels to PfEBA-175 were maintained for a period of time, and in a way dependent on age, seasonality and infection status. These findings suggest that increasing antibody response to clinical malaria may consequently boost antibody memory responses in individuals with malaria (Akpogheneta et al., 2008; Akpogheneta et al., 2010). However, some studies reported failed association between antibody levels to PfEBA-175 and protection against disease (Fowkes et al., 2010; John et al., 2005; Noland et al., 2008; Osier et al., 2008). These observations could be due to differences in study protocols (Fowkes et al., 2010), malaria exposure and antibody responses in the different malaria endemic settings (Kinyanjui et al., 2003).

For the other EBAs, it has been shown that antibody responses to PfEBA-140 RII and RII-V could play a role in protection against malaria, but antibodies against PfEBA-181 RIII-V have not been associated with protection (Richards et al., 2010). Naturally acquired antibodies against PfEBA140 was shown to be surprisingly inhibitory, compared to the other EBAs, when functional assays were applied. Subclass responses to the PfEBAs was in one study predominantly of IgG1 and IgG3 subclasses (Ford et al., 2007; Okenu et al., 2000; Persson et al., 2013). IgG3 was reported to be more protective than IgG1 against PfEBAs (Richards et al., 2010).

In conclusion, the antibodies against PfEBAs could have an important role in protection and invasion inhibition, and as result control parasitemia burden and decrease morbidity cases (Persson et al., 2013).

1.8.2.5 ANTIBODIES AGAINST PfRhs

There is limited knowledge regarding the antibody responses to PfRh antigens. Antibody responses to PfRh2a and PfRh2b have been reported to be associated with protection from clinical disease. A study in Papua New Guinea reported a significant correlation between high levels of antibodies against PfRh2a/b and reduced risk of symptomatic malaria. Furthermore, cytophilic IgG1 and IgG3 were the predominating responses in this study (Reiling et al., 2010). High levels of antibodies against PfRh4 have been shown to be strongly associated with a reduced malaria risk (Reiling et al., 2010). These findings suggest that PfRh2a/2b and PfRh4 could be important targets of protective immunity and potential vaccine candidates, but further studies are required to understand their role in immunity against malaria.

1.9 HUMAN RED BLOOD CELL POLYMORPHIMS AND MALARIA

Genetic factors are major determinants to consider in child survival in malaria endemic countries. In sub-Saharan Africa, the red blood cell polymorphisms (hemoglobin S (HbAS), α-thalassemia, β- thalassemia, glucose-6-phosphate-dehydrogenase (G6PD) deficiency, hemogloblin E, and ovalocytosis) have protective effects against severe *P.falciparum* malaria, and epidemiological evidence links the distribution of these polymorphisms to areas with high malaria endemicity (May et al., 2007; Rowe et al., 2007; Williams et al., 2005c). In this thesis we studied the protective effects of HbAS in malaria, and the following section will focus on HbAS and malaria.

1.9.1 MALARIA PROTECTION BY HbAS

Hemoglobin S in the heterozygous form (HbAS, sickle cell trait) has been reported to have a protective effect against malaria (Aidoo et al., 2002; Williams et al., 2005b). A single point mutation (β_6 Glu to Val) in the sixth codon of the β chain results in HbAS (Ingram, 1957). In sub-Saharan Africa this is thought to contribute to a survival benefit. The exact mechanism of how HbAS confers protection in malaria is still unclear. Proposed mechanisms include: I) reduced parasite invasion and/or growth in RBC (HbAS) (Friedman et al., 1978; Pasvol et al., 1978)., II) increased phagocytosis of pRBC (HbAS) by macrophages, and III) impaired cytoadherence of pRBC (HbAS) to microvascular endothelial and other cells (Bunn, 2013; Fairhurst et al., 2005; Williams, 2006).

It has been reported that individuals with HbAS have 70-90% protection against severe malaria (Aidoo et al., 2002; Jallow et al., 2009; May et al., 2007) and 50% protection against uncomplicated malaria compared to individuals with HbAA (Crompton et al., 2008; Williams et al., 2005b). The protection against uncomplicated malaria has been shown to increase with age (Williams et al., 2005a). Even though the above studies showed that HbAS protected against uncomplicated malaria, it has not been consistent in other studies (Allen et al., 1992; Lell et al., 1999). Numerous studies showed individuals with HbAS to have lower parasite densities compared to HbAA, which might indicate that HbAS enhance control of infection once parasitemia is established (Aidoo et al., 2002; Crompton et al., 2008; May et al., 2007; Williams et al., 2005b). The underlying mechanism of the antibody response mediated protection and the impact of Hb variants on protective antibody response has not been

completely solved. It has been hypothesized that both an innate and an acquired mechanism mediate protection that is conferred by HbAS.

1.10 MALARIA VACCINES

A major public health intervention that has had an enormous impact on the global health is vaccination. It is used to control and eradicate diseases such as smallpox, polio, rabies, diphtheria, tetanus, yellow fever, measles, mumps, rubella and hepatitis B.

At the moment several malaria control interventions are being used to reduce the burden of malaria. The malaria interventions are broadly divided into I) vector control (indoor residual spraying (IRS), insecticide treated nets (ITN), and larval control, II) intermittent preventative therapy (IPT) and III) immediate diagnosis and treatment (WHO, 2012). These interventions have contributed to effective reduction of *P.falciparum* malaria transmission and mortality (WHO, 2010, 2012). However, the *P. falciparum* parasite is highly complex and adaptable with emerging drug resistance (Dondorp et al., 2009). Based on this, developing a safe, effective and affordable vaccine would provide a much-needed tool to combat the morbidity and mortality of malaria.

The vaccine studies in malaria target the sporozoite, pre-erythrocytic (liver stage), erythrocytic (blood stage) and sexual stages. Comprehensive immuno-epidemiological studies have provided insight to the best candidate antigens to be included in vaccine development. One needs to remember that natural immunity targets a wider range of *P. falciparum* antigens and no specific antigens appears to be especially important to providing protection (Marsh and Kinyanjui, 2006). Most malaria antigens selected for vaccine candidates are targets of natural immunity, and display significant genetic polymorphisms. Additionally, the difficulties vaccine developers face with *P.falciparum* is that no adequate rodent model exists.

A new formulation of *P.falciparum* cirumsporozoite protein, known as RTS,S, a hybrid protein particle formulated in a multi-component adjuvant named AS01, showed promising results in sporozoite challenge studies (Stoute et al., 1997), and this was then moved onto field testing in West Africa (Bojand Lancet 2001). At this time the importance of cellular immunity in protection against the liver stage malaria had been confirmed in animal studies (Schofield et al., 1987). RTS,S/ showed 30-50% sterile efficacy across a series of sporozoite challenges in volunteers (Kester et al., 2009) and demonstrated promising ability to induce high antibody levels, that target the conserved regions of the CS protein, and in some settings the level of the antibody responses correlated with protection against infection or disease (Moorthy MJ 2009). The RTS,S/AS01 challenge studies achieved a level efficacy that was a clear step forward for the field, and has undergone a series of phase I and II clinical trials in several African countries (Aide et al., 2010; Olotu et al., 2011). However, a recent report demonstrated that the efficacy of RTS,S/AS01 vaccine after 4 year period was only 16.8 % against *P. falciparum* malaria among children vaccinated at 5 to 17 months of age in malaria endemic areas (Olotu et al., 2011).

Other vaccine approaches are a whole parasite vaccine such as developing genetically attenuated parasites incapable to reach the liver stage. Yet the lack of irradiation in some cases raises issues of safety and the risk of developing the disease (Vaughan et al., 2010). Blood stage whole parasite vaccines have shown potential efficacy but problems arise such as the risk of growing large number of parasites in the blood or risk of unknown infections in donated human blood (Pombo et al., 2002). Vector vaccines are another approach in vaccine development, and

aim mainly to induce cellular immunity against liver stage malaria, and are being assessed clinically (Reyes-Sandoval et al., 2010).

The progress with blood stage vaccines has been slow yielding mixed results (Goodman and Draper, 2010). A number of blood stage vaccine candidates have undergone clinical testing but not accomplished good protective efficacy against clinical malaria. The vaccines are based on a few candidate antigens; MSP1, MSP2, MSP3 and AMA1 (Dicko et al., 2008; Malkin et al., 2008; Ogutu et al., 2009; Sirima et al., 2007). Another blood stage vaccine candidate is PfEBA-175. A potential PfEBA-175 II vaccine was tested in malaria naïve subjects in the USA, and was shown to be safe and immunogenic. However, further studies are required in individuals living in malaria endemic areas (El Sahly et al., 2010). There are three challenges in blood stage vaccine development; expressing large antigens with the correct conformation, the modest antibody response and the extensive polymorphisms in many leading candidates.

The struggle with blood stage vaccines has shifted the enthusiasm to the field of transmission-blocking vaccine development. The leading vaccine candidates are Pfs25/ Ps48/45 and Pfs230 (Wu et al., 2008). Another approach is to use antigens from the *Anopheles* midgut wall, aminopeptidase APN1, as a suitable transmission-blocking vaccine component component (Dinglasan et al., 2013). These vaccine candidates have a particular advantage since they might be effective against more than one species of malaria, and been also considered as components for multi-stage vaccines. Consequently, this has renewed interest in development of mosquito stage vaccine candidates.

An effective malaria vaccine is most likely to contain a combination of antigens from the same stage of the parasite´s life cycle or from different stages. Several combination vaccines have been developed and tested, as for example AMA-1 and MSP-1, CSP and AMA-1 and MSP-1, MSP-2 and RESA (Genton, 2008; Malkin et al., 2008). However, there is limited evidence from human studies that these combinations have greater efficiency than single-antigen vaccines, but animal studies indicate that this might still be the case. Nevertheless, it is of great importance to find the best combination vaccine to improve the efficacy.

2 SCOPE OF THE THESIS

The overall objective of the work presented in this thesis was to increase our understanding of acquired immunity to the *P. falciparum* merozoite antigens in children and adults, and to identify functional antibody responses in uncomplicated and severe malaria that may play a role in the development of protective immunity. The merozoite invasion, growth and rosetting in the context of uncomplicated and severe malaria were also investigated.

SPECIFIC AIMS

PAPER I
To investigate the quantitative and qualitative antibody responses to the merozoite antigens, and the multiplicity of infection (MOI) in children with uncomplicated or severe *P. falciparum* malaria.

PAPER II
To study the role of merozoite invasion, parasite growth and rosetting in uncomplicated and severe *P. falciparum* malaria.

PAPER III
To investigate the natural acquired immunity against *P. falciparum* merozoite antigens over one-year period in children and adults, and the protective effects of hemoglobin S.

3 EXPERIMENTAL PROCEDURES

3.1 STUDY POPULATIONS

The studies included in this thesis work were conducted in populations living in *P. falciparum* endemic areas: north of Uganda (**Paper I** and **II**) and southwest of Nigeria (**Paper III**).

3.1.1 UGANDA (PAPER I and II)

Papers I and II were based on a cross-sectional study that included a total of 93 children under the age of five, with active *P. falciparum* infection. During 2002 children were recruited at the district hospital in Apac, Northern Uganda, a holo-endemic area having a very high entomological inoculation rate (EIR) of 1 500 per year (Okello et al., 2006; Yeka et al., 2005). Active *P. falciparum* infection was diagnosed by Giemsa blood smears and clinical examinations. The children were grouped according to the WHO guidelines (WHO, 2000a), and the modified Blantyre score (Molyneux et al., 1989). Fifty-one children were classified as having severe malaria and 42 children as having uncomplicated malaria. The severe malaria cases were further classified into sub-groups; respiratory distress (n=28), cerebral malaria (n=7), malaria NUD (n=10) (including convulsions, prostration, hyperparasitemia and hyperpyrexia), circulatory collapse (n=3), severe anaemia (n=2), one patient with cerebral malaria, severe anaemia, and respiratory distress (n=1). Paper I included 85 children (6 months to 3 years old), severe malaria (n= 46) and uncomplicated malaria (n=39). Paper II included 76 children under the age of five, with severe malaria (n=36) and uncomplicated malaria (n=40).

3.1.2 NIGERIA (PAPER III)

Paper III was based on a longitudinal study conducted in a rural town, Igbo-Ora in southwestern Nigeria. Malaria is endemic with transmission peaks during the rainy season (April to October), with mean EIR of 131 per year (Noutcha and Anumdu, 2009). During the study from July 2009-July 2010, there initially 200 individuals ranging from 5-70 years old enrolled. Venous blood samples were collected at baseline for blood group typing, *P. falciparum* genotyping and immunological studies. Thick and thin smears were made for parasitological investigations. The participants were followed up monthly, for a period of 12 months, checking their health status and the presence of malaria infection. Only participants who were permanent residents were included in the study. From 200 individuals 40 were randomly selected to be included in Paper III.

3.2 ETHICAL CONSIDERATION

Ethical approvals for the human participants in Paper I-III included in this thesis were obtained from the ethical research committees in Uganda, Nigeria and Karolinska Institutet, Sweden. Written informed consents were obtained from adult participants and parents or guardians of all children.

3.3 PARASITES AND *IN VITRO* CULTURES CONDITIONS

P. falciparum laboratory and clinical parasite isolates were included in Papers I-II. All laboratory strains and clinical isolates were cultivated using standard methods with modifications (Moll K, 2008; Trager and Jensen, 1976). In brief all *in vitro* adapted Uganda isolates were cultivated in a gas mixture of 90% NO_2, 5% O_2 and 5% CO_2, with constant and shaking instead of static candle-jar technique. This improved method has shown to facilitate the establishment of fresh clinical isolates to *in vitro* cultivation and to reduce the number of multiple infected RBCs.

Parasites were kept synchronized using 5 % sorbitol (w/v) treatment. The parasitemia was counted and the rosetting rate was determined by calculating the number of trophozoite pRBCs within rosettes, relative to the total number of trophozoite pRBC in the culture. A rosette was defined as at least two unparasitizd RBCs bound to one pRBC. The rosetting phenotype was maintained with Ficoll enrichment.

3.4 INVASION INHIBITION ASSAY

The method described previously (Persson et al., 2006; Persson et al., 2008) allowed us to study the invasion inhibitory activities in patient plasma. Two Ugandan *P. falciparum* clinical isolates (UAM37, from a patient with uncomplicated malaria, and UAS31, from a severe malaria patient) were chosen as representative of clinical isolates in Paper I. Isolates were cultured *in vitro* in AB+ non-immune Swedish serum and gassed with 90% NO_2, 5% O_2 and 5% CO_2, and placed in a shaking incubator. In brief, parasites were synchronized (5% sorbitol, v/w) before starting the assay and used when the majority of the parasites had reached a late-pigmented trophozoite stage. 45 µl of parasite suspension and 5 µl of dialyzed test plasma were added to each well in duplicates. Plates were incubated in sealed, humidified and gassed box for 48 hours at 37 ^{0}C. Parasitemia was estimated using hydroethidine (10 µg/ml; Sigma Aldrich) in a flow cytometer (FACS Scan; BD). Parasite invasion for each sample was calculated relative to control samples (invasion in presence of dialyzed Swedish plasma). Parasite invasion was considered 100 %, if there was no invasion inhibitory activity in the added plasma sample.

3.5 INVASION INTO ENZYME TREATED RED BLOOD CELLS

To further characterize the clinical *P. falciparum* parasites, 21 isolates (11 uncomplicated and 10 severe) were examined for their RBC receptor specificities in invasion assays in Paper II. Human O+ RBCs were washed with RPMI 1640 (Gibco, Invitrogen), and subsequently treated with neuraminidase (62.5 uM/ml; Sigma), trypsin (1mg/ml; Sigma) and chymotrypsin (1 mg/ml; Sigma) for 45 minutes at 37 ^{0}C with periodic shaking. Control RBCs were only treated with RPMI 1640. After incubation the RBCs were washed once with RPMI 1640 containing 20% human serum and twice with RPMI 1640 containing 10% human serum to inhibit enzyme activity (Persson et al., 2008). Late-pigmented trophozoite to schizont stages were enriched using magnetic bead column separation (Miltenyi Biotec, Germany). Invasion assays were performed in 96 U-bottom culture plates with a total of 50 µl of parasite suspension at 0.5–1.0% parasitemia and enzyme treated RBCs. All samples were run in triplicate. Plates were incubated in a gassed box for 48 hours at 37 ^{0}C. Parasitemia was estimated using hydroethidine (10 ug/ml; Sigma) in a flow cytometry (FACS Scan; BD) after 48 hours.

Invasion inhibition by enzyme treatment was determined as [1 - (proportion of enzyme-treated cells invaded/proportion of untreated cells invaded)] x 100. Results presented were comparative with control treated cells.

3.6 RECOMBINANT MEROZOITE ANTIGENS

A panel of *P. falciparum* merozoite antigens were selected and included in Paper I and III. The antigens were selected based on extensive studies suggesting them as potential candidates for vaccine development. All antigens were expressed in *Escherichia coli*, including following: the whole ectodomain of the *P. falciparum* D10 allelic form of PfAMA1 (AMA1-D10) (Hodder et al., 2001); regions III to V; PfEBA140 (3D7; aa; 770 to 1064), PfEBA175 (3D7; aa; 761 to 1298), and PfEBA181 (3D7; aa; 769 to 1365) (Richards et al., 2010); and PfRh2A9 (3D7; aa; 2027 to 2533) (Reiling et al., 2010) and PfRh4A3 (aa; 1160 to 1370) (Persson et al., 2008; Stubbs et al., 2005). The MSP2 proteins corresponding to the FC27 and 3D7 gene sequences (Adda et al., 2009)were expressed as described previously. MSP1-19 from the 3D7 sequence was expressed as described previously) (Dutta et al., 2005). The recombinant antigens were all GST-tagged.

3.7 MEASURING TOTAL IMMUNOGLOBULIN G (IGG) AGAINGST RECOMBINANT MEROZOITE ANTIGENS BY ELISA

Antibody responses to *P.falciparum* merozoite antigens in patient plasma were assessed using ELISA. Total IgG levels against a panel of merozoite antigens (PfEBA140, PfEBA175, PfEBA181, PfRh2, PfRh4, MSP1-19, MSP2-3D7, MSP2-FC27, and PfAMA1) were measured using previously published ELISA protocols (Persson et al., 2008; Reddy et al., 2012).

3.8 MEASURING SUBCLASS IG1-IGG4 AGAINGST RECOMBINANT MEROZOITE ANTIGENS BY ELISA

This assay was adapted and optimized to quantify subclass antibodies against merozoite antigens PfEBA-175 and PfRh2 in patient plasma as described in Paper III. In brief the subclass reactivity was measured as follows: flat bottom 96-well plates (Nunc-immunoplate, Thermo Scientific) were coated with 1 μg/ml recombinant antigen in coating buffer (15 mM Na_2CO_3 and 35 mM $NaHCO_3$; pH 9.6) and incubated at 4 ^{0}C overnight. The plates were washed three times after incubation with blocking medium or antibodies. Plasma samples were diluted to 1:25 in 5% skimmed milk in PBS- Tween 20, and incubated for 1.5 hours at 37°C. Mouse anti-human antibodies (IgG1 A10630, IgG2 05-3500, IgG3 05-3600, IgG4 A10651 from Invitrogen Corporation, CA) were diluted in 5% skimmed milk in PBS-Tween 20 (1:500 for IgG1 and IgG3, and 1:250 for IgG2 and IgG4, dilutions chosen after optimization), and incubated for 1.5 hours at 37 ^{0}C. Followed by antibody detection with goat anti-mouse IgG (H+L) horseradish peroxides (HRP) (G21040, Invitrogen Corporation, CA) diluted at 1:500 for IgG1 and IgG3, and at 1:1000 for IgG2 and IgG4, and incubated for 1.5 hours at 37 ^{0}C. Antibody reactivity was detected with a azino-bis(3-ethylbenthiazoline-6-sulfonic acid) (ABTS) tablet (Sigma Aldrich) dissolved in phosphate citrate buffer pH 5.0 (Sigma Aldrich); 30% H_20_2 was added just prior to use. Plates were incubated at room temperature for 1 hour and optical density (OD) was read at 414 nm. All assays were run in duplicate and OD was subtracted for non-specific binding of the

fusion tag, GST. Positive (pools of immune samples) and negative (Swedish non-immune) controls were included in all plates.

3.9 SURFACE PLASMON RESONANCE

To estimate the affinity of antibodies in plasma, binding to merozoite antigens in Paper I, the recombinant MSP2-3D7, MSP2-Fc27 and PfAMA1-D10 were bound to CM5 chips in Surface Plasmon Resonance assays (SPR) (Biacore 3000, Uppsala, Sweden) as described previously (Reddy et al., 2012). In brief, one lane was used as a control lane. Around 1000 response units of each protein was bound to the chip. Plasma samples in at least two different dilutions (used as "internal controls", since the off rate k^d should be the same independent of concentration) were flowed over the antigen-coated surfaces, and the k^d was measured in real time. When antibody levels were very high, the samples were diluted further until similar levels of response units were accomplished. For every 20 samples, a control sample (a pool of adult immune plasma) was run to check that there was still enough protein bound to the chip to allow for measurement of reproducible k^d values, and this was also used as a control between different chips. Swedish non-immune sera were used to determine the cut-off to be considered as background level (=100 RU). Data was analyzed using BIAevaluation 4.1 software (Langmuir binding model).

3.10 AMMONIUM THIOCYANATE (NH_4SCN)-ELISA

To estimate the strength of the antibody binding in Paper I, increasing concentrations of NH_4SCN were added to ELISAs for the MSP2-3D7 and MSP2-FC27 proteins as described previously (Reddy et al., 2012). The affinity index was calculated from the absorbance reading in the presence of increasing concentrations of NH_4SCN, which were converted to percentage of total bound antibody (in the absence of NH_4SCN). The index was calculated from the molar concentration of NH_4SCN required to reduce the initial absorbance by 50% (Arias-Bouda et al., 2003).

3.11 DETERMINATION OF *P.FALCIPARUM* GENETIC DIVERSITY

27 uncomplicated and 29 severe *P. falciparum* clinical cases were randomly selected from the clinical samples in Paper I for genotyping. A nested PCR was used to study the genetic markers for the MSP2 alleles (Fc27 and 3D7), for CSP, GLURP, and MSP1 alleles (K1, MAD20 and RO33). The primers and experimental conditions used have been described previously (Snounou, 2002), with some modifications (Ribacke et al., 2007). Multiplicity of infection (MOI) for each isolate was determined based on the highest number of allelic variants observed for a given genetic marker.

4 RESULTS AND DISCUSSION

Results are discussed in detail in each respective study included in this thesis (Paper I-III). The original results and interpretations are also summarized here.

4.1 PAPER I

" Acquired antibodies to merozoite antigens in children from Uganda with uncomplicated or severe *Plasmodium falciparum* Malaria"

Antibodies against several *P.falciparum* merozoite antigens have been found to be associated with protective immunity but very few studies have investigated the functional properties of acquired antibodies, or examined the role of antibodies to merozoite antigens in immunity to severe malaria in young children.

In Paper I, we investigated the quantitative and qualitative differences in antibody responses in plasma against a panel of merozoite antigens, using a set of assays including ELISA, Invasion Inhibition Assays (IIA), NH_4SCN-ELISA and Surface Plasmon Resonance (SPR) to identify antibody responses that may play a role in protective immunity, and evaluate which assay(s) are best predictors of immune differences between severe or uncomplicated malaria. Additionally, we studied the MOI in both the uncomplicated and severe malaria group.

The IgG levels of antibodies against PfEBA181, MSP2-3D7, MSP2-Fc27 and PfAMA1 were significantly higher in children with uncomplicated compared to severe malaria. On the other hand, there was no significant difference in IgG levels of antibodies against PfEBA175, PfEBA140, MSP1, PfRh2 and PfRh4 in uncomplicated compared to severe malaria. Earlier studies have shown associations between high levels of antibodies against different merozoite antigens and protection against symptomatic malaria in general (severe malaria was not addressed in these studies), while other studies have defined high antibody levels as a marker of exposure (Osier et al., 2008; Polley et al., 2004; Richards et al., 2010). Based on previous findings showing inconsistencies in the correlations of antibody responses to recombinant antigens and protection from malaria using ELISA (McCallum et al., 2008), we further investigated the affinity properties of antibodies present in patients with uncomplicated or severe malaria, with SPR and the qualitative (functional) properties with IIA.

In the first approach, we used an SPR method which allowed us to study antibody affinity under flow in real time (Hearty et al., 2010). Two allelic variants of the highly unstructured MSP2 protein, MSP2-Fc27 and MSP2-3D7, as well as PfAMA1 were selected for this purpose. Antibodies against PfAMA1 showed the highest affinity (i.e. lower k^d values), while antibodies against MSP2-3D7 had lower affinity, and antibodies against MSP2-Fc27 had the lowest affinity. For all three antigens, there was a trend of higher affinity among the uncomplicated malaria cases, significant only for PfAMA1. These findings could indicate the importance of antibodies against PfAMA1 in this study group for a role in protection against severe malaria. These results are supported by a recent study using SPR that showed that individuals with high affinity antibodies directed against MSP2-3D7 had a prolonged time before developing clinical malaria, indicating that presence of high affinity antibodies may be important in protection against malaria (Reddy et al., 2012).

In our second approach, we studied the inhibitory activities in plasma, *in vitro*, from children with uncomplicated or severe malaria, using IIA. All plasma samples from the

children were tested against two clinical parasite isolates, UAM37 and UAS31. The parasite UAM37 originated from a patient with uncomplicated malaria while UAS31 came from a patient with severe malaria. There was a trend of decreased invasion (58%) when plasma from children with uncomplicated malaria patients were used against UAS31, compared to severe malaria (70% invasion), but the difference was not significant. Furthermore, microscope and flow cytometry revealed that the major part of the invasion inhibition was found within the first hours of the parasite´s life cycle. We could clearly observe that both clinical isolates (UAM37 and UAS31) showed significantly higher invasion in the presence of plasma from children with high initial parasitemia, indicating a lack of inhibitory antibodies in their plasma. However, we did not see any correlation between initial parasitemia and the presence of either IgG or IgM with any of the merozoite antigens tested by by ELISA or SPR.

As a next step, the measurements of antibody responses were compared with the results of the functional assays. The most extensive analysis was carried out for the MSP2-3D7 and MSP2-Fc27 antigens, for which we had access to two different allelic forms of the proteins. The IgG ELISA results for MSP2-3D7 and MSP2-Fc27 correlated very well with the NH_4SCN-ELISA, which is not surprising since both methods are very similar. MSP2 contains both conserved and variable domains, and there might be a certain degree of overlap in antibody responses between the two allelic variants due to antibodies reacting against the same epitopes. Moreover, a significant correlation between parasite invasion of the two clinical isolates and presence of IgG/IgM in ELISA could be seen, between the results of the IIA on UAS31 and the IgG ELISA response against MSP2-Fc27, as well as between the for UAM37 and the PfEBA175 ELISA results, for both IIA on UAS31 and UAM37 and PfRh2 ELISA. Furthermore, it was observed that plasma with higher levels of antibodies had an inhibitory effect on parasite invasion in IIA. For PfAMA1, we observed the most interesting correlation in antibody response in the investigated functional assays. We saw a significant correlation of decreased UAS31 and UAM37 parasite invasion in IIA with increasing anti-PfAMA1-IgG in ELISA. We could also see a correlation between high levels of IgG in ELISA and high antibody affinity in SPR. For the other recombinant proteins used in IgG ELISA (PfEBA140, PfEBA181, PfRh4, MSP2-3D7 and MSP1), no significant correlations could be seen with IIA results. Several of the IgG ELISA results correlated with each other, but only PfAMA1 stood out as showing best correlations between methods (ELISA, IIA and SPR), compared to any of the other proteins.

We also investigated the genotypes of the Ugandan isolates. The majority of analyzed samples were multi-clonal, including presence of both MSP2-FC27 and MSP2-3D7 alleles. CSP, GLURP, and MSP1 (K1, MAD20, and RO33) and MSP2 (FC27 and 3D7) were analyzed and they showed a significantly larger heterogeneity in patients with uncomplicated compared to severe malaria for MSP1-K1 and MSP2-3D7. The total MOI in uncomplicated cases was significantly different as compared to severe cases, which has been previously shown in highly endemic settings (Farnert et al., 2009). Patients often harbour many variants of parasites at the same time (Arnot, 1998; Hoffmann et al., 2001) and there is conflicting data on whether multi-clonal infections are associated with the outcome of the infection (al-Yaman et al., 1997). There was a significant positive correlation between MOI and IgG in ELISA against the following merozoite antigens: MSP2-FC27, PfAMA1, PfEBA175, PfEBA-181 and PfRh2. A higher MOI correlated weakly with lower affinity of antibodies (in SPR) for MSP2-FC27 and MSP2-3D7. In addition, age and the presence of IgM in ELISA against MSP2-FC27 correlated positively as did age and IgG in ELISA against MSP2-FC27 and PfAMA1. Older children had higher levels of antibodies and they also had a higher antibody affinity against the MSP2 antigens measured by NH4SCN ELISAs, but not with SPR.

Our study is the first to show that by using a combination of assays we could increase the understanding of the immunological responses in children with uncomplicated or severe *P.falciparum* malaria. No single method has shown to be fully predictive of immunity (Bouharoun-Tayoun et al., 1990; Dent et al., 2008; Egan et al., 1996; Hodder et al., 2001), and the most commonly used method, ELISA, is a static method that does not reflect the function of antibodies. In our combination of assays, we found that the presence of antibodies against PfAMA1 (in ELISA) correlated to invasion for both tested clinical isolates, and that these antibodies against PfAMA1 in ELISA also correlated to affinity SPR results, indicating that this protein might be targeted by functionally important antibodies with high affinity that could contribute to protection from severe malaria in this study group. Our observations are consistent with recent studies from Uganda (Greenhouse et al., 2011; Keh et al., 2012), which showed a strong association between antibodies against PfAMA1 and protection against malaria. Presence of antibodies against PfAMA1 has been shown to be associated with reduced incidence of malaria (Polley et al., 2004), and individuals living in malaria endemic areas have increased levels of anti-PfAMA1 antibodies, which can be strongly inhibitory to parasite invasion (Courtin et al., 2009; Hodder et al., 2001; Nair et al., 2002). Furthermore, antibodies against MSP2-3D7, but not against MSP2-FC27, have been associated with protection against malaria (al-Yaman et al., 1997), and for being more important in protection against severe malaria (Iriemenam et al., 2009). These previous findings are corroborated by our results that show a higher affinity of antibodies against the 3D7 allelic variant (compared to FC27) in SPR. Interestingly, the only assay that positively correlated with initial parasitemia in the patient was the IIA, which emphasis the importance of using functional assays.

4.2 PAPER II

"Improved *in vitro* culture of *Plasmodium falciparum* permits establishment of clinical isolates with preserved multiplication, invasion and rosetting phenotypes"

Cytoadherence and rosetting in *P. falciparum* cause excessive micro-vascular sequestration, obstruction of blood flow and severe disease (Carlson et al., 1990; Rowe et al., 1995). The peripheral parasitemia and the sequestrated biomass of *P.falciparum* are higher in individuals with severe malaria than individuals with uncomplicated malaria. The study of rosetting, multiplication and RBC invasion has been limited by difficulties in adapting fresh isolates and cryo-preserved clinical isolates to *in vitro* cultures. The problems are poor outgrowth, low multiplication rates, loss of rosetting and cytoadherence phenotypes due to lack of appropriate environment and specific host factors.

In Paper II, we evaluated the optimal *in vitro* growth conditions for the parasites that would mimic the environment of the human microvasculature. We used long-term propagated parasites not selected for rosetting or cytoadhesion (HB3, F32, 3D7AH1, FCR3, R29 and TM284), parasite clones selected by micromanipulation for high or low rosetting (FCR3S1.2, FCR3S1.6, TM284S2 and3D7S8.4) and 76 *P.falciparum* clinical isolates collected from children with uncomplicated or severe malaria in Apac, Uganda (also used in Paper I).

A systematic evaluation was performed to establish 100% of the cryo-preserved clinical isolates with minimal parasite mass loss and preservation of the phenotype. Four different *in vitro* growth conditions were studied, and the cultivation involving growth in suspension on an orbital shaker (50 rev/min) with the use of a fixed gas composition (5% O2, 5% CO_2 and 90% N_2) to maintain a stable micro-aerophilic environment was found to be

preferred compared to static cultivation with the same gas or the candle-jar method. 100% of isolates were established, with reduced multiple infected RBC, high multiplication rate and preserved capacity to form rosettes over time.

We further investigated the rosetting in the parasites stains and clinical isolates, and we found that the dependence of rosetting in the presence or absence of serum varied in different parasites strains. The FCR3S1.2 pRBCs did not show any rosettes when grown in absence of human serum, while the rosetting rate was high, around 80%, in the presence of human serum. Switching of culture conditions rapidly re-established the rosetting phenotype in the presence of human serum, while it was lost by a change to conditions without human serum. Surface expression of PfEMP1 in FCR3S1.2 was significantly lower in pRBCs grown in the presence of Albumax as compared to human serum. Furthermore, expression of PfEMP1 was only detected between 22 h and 34 h post infection (pi) in Albumax while PfEMP1 in pRBCs grown with human serum was expressed between 18 h and 42 h pi. These findings argue for that serum components are necessary for correct display of PfEMP1 on the pRBC surface. We also observed that all 76 clinical isolates formed rosettes during schizogony, varying from 26 to 88 %. Invasion of bound uninfected RBC at the time of rupture was frequently observed. Rosetting could be associated to the parasite's ability to invade new RBC. Parasites previously selected for the rosetting phenotype at trophozoite stage showed high rosetting rates during schizogony.

Of the 76 clinical isolates, 36 originated from children with severe malaria and 40 from children with uncomplicated disease. When the groups were compared, we found that the severe malaria parasites multiplied and rosetted (both at trophozoite and schizont stage) at significantly higher rates. Interestingly, positive and significant correlations of the multiplication rates of individual isolates to the original peripheral parasitemia at the time of blood collection were also observed. The ability of clinical isolates to multiply correlated to the rosetting rates of both trophozoite and schizont-infected RBCs.

We further investigated if the correlation between rosetting and multiplication rate was due to facilitated invasion during rosette formation. Parasites, to which specific antibodies towards the expressed PfEMP1variant were available, were grown in presence and absence of those antibodies. However, no growth advantages and no correlation between rosetting and multiplication was observed. To further characterize the Ugandan clinical isolates, 21 (11 uncomplicated and 10 severe) of the 76 clinical isolates were studied for different invasion pathways in merozoite invasion assays. The RBCs were treated with trypsin, chymotrypsin or neuraminidase before merozoite invasion assays. The majority of isolates were neuraminidase resistant, trypsin and chymotrypsin sensitive. Parasite invasion into the enzyme treated RBC differed slightly between the uncomplicated and severe group. Isolates from children with uncomplicated malaria showed significantly higher sensitivity to trypsin and chymotrypsin. There was no difference in sensitivity towards neuraminidase treatment of erythrocytes observed between the groups. The Ugandan clinical isolates to a large extent use sialic acid independent pathways.

In this study, we established improved *in vitro* culture conditions in suspension and a controlled micro-aerophilic environment for cryo-preserved clinical *P.falciparum* isolates, which allowed us to study multiplication rates, rosetting phenotypes and merozoite invasion. We also found it to be the preferred conditions for both fresh and established parasites for maintaining their phenotypes. This study further emphasizes the importance of human serum during the cultivation of *P. falciparum* isolates and laboratory strains to guarantee the display of the parasite's adhesive phenotype and correct expression of PfEMP1. The role of PfEMP1 in the close interaction between infected and uninfected RBCs in rosettes may facilitate merozoite invasion by bridging molecules or merely by being in the proximity of the pRBC during

schizont rupture. The surprising presence in all fresh isolates of high levels of schizont rosettes could suggest that rosetting must have an important biological function and can give an advantage in survival/growth. The peripheral parasitemia, the level of rosetting and the rate of multiplication correlated positively to one another for individual isolates and were all found to be higher among isolates from children with severe malaria than from children with uncomplicated malaria. This suggests that rosetting may contribute to a higher parasite burden *in vivo*. In addition, other traits than rosetting such as the rate of replication, number of formed merozoites and the invasion ligand repertoire are also likely to contribute to the superior growth of some, but not all, parasites. In this case, sialic acid independent invasion pathways were mainly used by these clinical isolates and this pathway is known to by used by the parasite ligand PfRh4. This is important information for future vaccine studies.

4.3 PAPER III

"Antibody responses against PfEBA175 and PfRh2 in naturally acquired immunity against *Plasmodium falciparum* malaria"

Acquired immunity to malaria develops after repeated exposure in individuals living in endemic areas (Doolan et al., 2009; Langhorne et al., 2008). In Paper III, we investigated the naturally acquired antibody responses to *P.falciparum* merozoite antigens in 40 children and adults in Igbo-Ora, a rural area of Nigeria, over a time period of almost one year (July 2009 to June 2010). The malaria protective effect of having hemoglobin S (HbAS), which is relatively common in this area, was also investigated. The total IgG and subclass IgG1-4 responses against PfEBA175 and PfRh2 during one year were investigated in Nigerian children and adults.

In both children and adults, the antibody responses (total IgG and subclass IgG1-IgG4) against PfEBA175 and PfRh2 increased from August with a peak in November followed by decline in the following months. In children and adults, the cytophilic IgG1 and IgG3 responses against PfEBA175 dominated and IgG2 and IgG4 responses against PfRh2 were also detected. Additionally, we found that the total IgG levels against PfEBA175 and PfRh2 showed a positivly significant correlation with each other. Furthermore, when the total IgG and subclass responses within PfEBA-175 were investigated and we observed that the total IgG responses significantly correlated with all four subclasses, and the subclasses significantly correlated with each other. While for PfRh2 we saw that total IgG significantly correlated with IgG1, and IgG2 significantly correlated with IgG3 and IgG4. We further looked into the acquired antibody responses in the context of age, and found that age significantly correlated to the levels of total IgG, IgG1 and IgG3 for PfEBA175.

Patient parasitemia was carefully monitored over the one-year study period. We observed that the parasitemia reached its peak in the month of October (mean: 749.7 parasites/μl), while the lowest peak was observed in May (mean: 3.7 parasites/μl). These findings could explain the peak of antibody levels arising just after the peak in parasitemia. Additionally, it is concordant with the transmission intensity being highest during the rainy season, which ends in October. There was no significant difference in parasitemia between children and adults.

We compared the antibody levels with parasitemia to determine if the antibody responses against PfEBA175 and PfRh2 correlated with control of parasitemia during the study period. We found that higher IgG1, IgG2 and IgG3 levels for PfEBA175 and total IgG for

PfRh2 correlated significantly with lower parasitemia. We observed that individuals with the highest quartiles of levels of total IgG against PfEBA175 or PfRh2 showed a correlation with reduction of parasitemia, and when individuals with high levels of both antibodies against PfEBA175 and PfRh2 were considered, they had lower parasitemia compared to those with raised levels of only one of the antibodies. These findings indicate that these antibodies are important for protection against malaria, and support previous studies showing that high levels of total IgG, IgG1 and IgG3 against PfEBA175 associate with protection from malaria (McCarra et al., 2011; Richards et al., 2010).

We further investigated the impact of HbAA and HbAS in protective effects against malaria. When all individuals in the study group were considered together we found that total IgG, IgG1 and IgG3 against PfEBA175 were significantly higher in individuals with HbAA compared to HbAS. For PfRh2, only the IgG1 response was significantly higher in HbAA. When we divided the cohort into two groups, children and adults, we found that children with HbAS had significantly higher levels of IgG, IgG1, IgG2 and IgG3 against PfEBA175 and higher levels of total IgG against PfRh2 compared to HbAA. On the other hand, the pattern in the adult group was the opposite with higher levels of IgG, IgG1 and IgG3 against EBA175 in HbAA individuals. Although there were very few samples in each group, these findings may give an idea of the role of HbAS and its malaria protective effects in this Nigerian population.

In this study we showed that the antibody response against PfEBA175 was more evident than against PfRh2 in this Nigerian population. This could be explained by the fact that the expression of PfRh2 can vary between parasites (Duraisingh et al., 2003b), or that the used recombinant antigen of PfEBA175 was more similar to the protein variant within parasite population in that area. The part of the protein that was used, region III-V of PfEBA175 (Richards et al., 2010) in our case, could also affect the results. The few studies that have investigated the subclass responses against merozoite antigens have concluded that IgG1 and IgG3 responses are predominant (Roussilhon et al., 2007; Stanisic et al., 2009). Our results are concordant with dominant IgG1 and IgG3 responses, however we also observed responses against IgG2 and IgG4. The quality of IgG2 and IgG4 could be of importance in the development of protective immunity, even at low levels. Furthermore, we observed higher levels of antibodies in children with HbAS that might have malaria protective effects. Previous studies in Nigeria (Fleming et al., 1979) and other African countries (Williams et al., 2005a) have shown that individuals with HbAS had lower parasite densities and lower prevalence of clinical malaria.

Another important finding in this study is that individuals with high levels of both total IgG against EBA175 and PfRh2 had a lower parasitemia during the one-year follow up, compared to the high single antibody response. This observation of combined antibody response against two antigens emphasises the importance of aiming at the use of combined antigen vaccines (Lopaticki et al., 2011; Richards et al., 2010).

5 CONCLUDING REMARKS AND FUTURE DIRECTIONS

The main goal for malaria research is to decrease the global malaria burden. Even though there has been great achievements in malaria control and treatment during the last decade, the mortality rate is around 15-20 % for severe malaria, even in modern and well-equipped hospitals, and many patients die without receiving the correct care. To develop a safe, effective and affordable anti-malarial vaccine would provide a much-needed tool to combat the morbidity and mortality malaria is responsible for in the world. It is known that individuals living in malaria endemic countries develop naturally acquired immunity after repeated exposure (Doolan et al., 2009; Langhorne et al., 2008). Furthermore, antibodies are an important component of acquired immunity, and it has been shown that passive transfer of antibodies from immune donors to individuals with *P.falciparum* infections reduces parasitemia and clinical symptoms (Cohen et al., 1969). It is of great importance to understand the underlying role of antibodies in the development of protective immunity against severe malaria. Based on the papers included in this thesis, the following conclusion can be drawn and future prospects proposed:

I. Children with uncomplicated malaria had acquired more antibodies against a selection of merozoite antigens (PfEBA-181, MSP-Fc27, MSP2-3D7 and PfAMA1) compared to children with severe malaria. Acquired antibodies against PfRh2 and PfAMA1 in ELISA correlated to parasite invasion in both clinical isolates in the invasion inhibition assay (IIA), and anti-PfAMA1-antibodies in ELISA correlated with increased anti-PfAMA1-antibody affinity in Surface plasmon resonance (SPR). Importantly, the only assay that correlated with initial parasitemia in the patient was the IIA. Both MSP2Fc27 and MSP23D7 allele variants were present in both groups, and considering total MOI of the parasite there was a higher number of genotypes in uncomplicated malaria. For future studies, in addition to ELISA, it is of importance to include a combination of functional assays such as IIA and SPR assays, to understand the development of protective immunological responses against malaria in individuals living in endemic countries. Additionally, vaccine studies including merozoite antigens should consider the difference in immunological responses between uncomplicated and severe malaria in their evaluations.

II. Optimal *in vitro* growth conditions were achieved, which allowed for phenotypic studies of clinical *P.falciparum* isolates taken directly from the patients. Presence of serum in the cultures was found to be essential for optimal surface presentation of PfEMP1, and for maintaining of rosettes. Children with severe malaria had higher peripheral parasitemias, higher rosetting levels and higher multiplication rates and these correlated positively with each other. The majority of clinical isolates used a sialic acid independent invasion pathway. Isolates from children with uncomplicated malaria showed significantly higher sensitivity to trypsin and chymotrypsin. Rosetting might facilitate successful merozoite invasion *in vivo*, hence could be the reason it is found to be associated with severe disease. Parasite invasion is central to parasite replication and virulence. Future studies should investigate alternative merozoite ligand expression in uncomplicated and severe malaria in the context pathogenesis.

III. The antibody response against PfEBA175 was more prominent than against PfRh2 in in both children and adults. Cytophilic IgG1 and IgG3 against PfEBA175 were the predominant antibody responses, but there was also some response for IgG2 and IgG4. Individuals with higher total IgG responses against both PfEBA175 and PfRh2 had lower parasitemia during the one-year study period. Higher antibody responses against merozoite antigens in children with HbAS might have protective effects against malaria. Future studies will include investigation of antibody responses against other relevant merozoite antigens, and evaluation of functional properties of these antibodies, such as the invasion/growth inhibitory effects and affinity in the context immunological responses during the study period. Additionally, to consider the red blood cells polymorphisms present in the study area, and their role in acquired immunity against malaria could be of great value for future vaccine studies.

6 ACKNOWLEDGEMENTS

I would like to thank everyone who has been part of this journey, both in the academic world and outside the science world. I would like to express my gratitude to:

My supervisor, **Kristina Persson**, for welcoming me into her group, and for her support and trust in me during the past years. Thank you for giving me the freedom to explore. Most of all thank you for giving me the opportunity to develop into an independent scientist.

My co-supervisor, **Mats Wahlgren**, for welcoming me into his lab and group. Thank you for your endless enthusiasm, support and good advice over the past years. Thank you for providing me a wonderful workplace in malaria research.

My co-supervsior, **Susanne Nylén Spoormaker**, for her guidance, support and encouragement over the past years. I can not thank you enough for all your help during the ups and downs. Thank you for always taking your time to sit down and talk. Tack så mycket för allt!

My mentor and good friend, **Nicolas "Nico" Joannin**. You have been part of this journey since day one. Thank you for all your support over these years. For always being there for me, either over coffee breaks or a Skype-talk even with the crazy time differences ;). I can not put in words how much I appreciate your friendship, encouragement and always pushing me to do better and most of all for believing in me. Merci beaucoup!!! ☺

My unofficial mentor and very dear friend, **Letusa Albrecht**, your friendship over the past years has been invaluable. Thank you for everything (I will not make a list ;)), especially your support and help during the past years. So many fun memories too; ABBA sing-a-long evenings, the dinners, gorilla trekking in Uganda, salsa dancing in Barcelona and sightseeing in New York. Thank you for being such a wonderful friend and I'm truly blessed to have you as a friend. I miss you!

The co-authors and friends in the lab that made this work possible; lab guru **Kirsten Moll**, **Johan Normark** and **Ulf Ribacke**. Thank you so much for your great collaborations and scientific input.

The collaborators in Uganda; **Professor Fred Kironde** and **Thomas Egwang**. Big thanks to **Moses Wasswa** and **Wannume Henry** for all your help in the lab. I learned so much from you! **Ritah Kayizzi**, for being such a great lab mate and friend during my stay in Kampala. Thank you for showing me the local highlights ☺

The collaborators in Australia, **Professor James Beeson**, **Linda Reiling** and **Christine Langer**. Thanks to **Michelle Boyle** for teaching me the merozoite assay!

The collaborators in Nigeria, **Professor Roseangela Nwuba** and **Tijani Muyideen Kolapo**, thank you for providing the valuable patient samples and for your scientific input over the past year.

Thanks to senior members of the Wahlgren´s group for great and stimulating discussions over the years; **Akira Kaneko** and **Qijun Chen**.

Past and present members of the Wahlgren´s group for creating such a wonderful and fun workplace (24/7) filled with scientific input, laughter and joy. **Davide Angeletti** for sharing this journey with me since day one, **Mia Palmkvist**, **Mattias Westman**, **Sandra Nilsson**, **Karin Blomqvist**, **Fingani Mphande**, **Johanna Breuer**, **Josea Rono**, **Sreenivasulu Basi Reddy, Isabella Dellacasa**, **Allan Lugaajju**, **Florence Urio**, **Caroline Rönneberg**, **Kim Brolin**, **Adrian Luscombe**, **Zach Darroch**, **Katja Obiego**, **Marcus Österbald**, **Emilie Loft, Suchi Goel**, **Reetesh Raj Akhouri**, **Thomas Geislinger**, **Chim Chan**, **Zulkarnain "Zul" Md Idris**, **Junhong "Hon" Ch´ng.**

I would like to express especial big thanks to; Miss Sunshine aka **Susanna "Su" Bächle**, thank you for all the wonderful climbing sessions (with bruises and cuts!), "fika", lunch and dinners at your place! Thank you for always being there when I needed your help, just to talk or to proof-read! Tack finaste Su för att du är en underbar vän! **Sriwipa "Jeaba" Chuangchaiya**, for being such a great friend in the lab and outside, for bringing so much laughter and joy the past years. I truly miss your "crazy" green thai chicken! Thank you for everything! The amazing molecular biology wizard, **Sherwin "Sher" Chan**, thank you so much your patience and always answering my endless questions about molecular biology. Thank you for all the good times, GPS moments and your wonderful BBQ skills! I will miss that! Miss Colombiana aka **Pilar Quintana**, big thanks for being my everyday support and being an awesome friend both in the lab and outside! Thank you for everything for the past years, so many good and fun times we shared. I will always value our friendship! I will miss you tremendously, my favourite Colombian! **Tijani Muyideen Kolapo**, thank you for being such brilliant scientist and supportive friend! Your unwavering positive view in research and in life during the many ups and downs is something I will always carry with me. I have tremendous respect for you as fellow researcher and human being. I cannot thank you enough for everything. Thank you for your endless optimism, support and your friendship!

Big thanks to our fantastic, both previous and present, lab coordinators, **Karolina Palmgren** and **Martina Löfstedt Javala**, for a wonderful administrative support and great company in the lab.

I would like to thank the malaria researchers in Stockholm and in Sweden. The SMN for the great seminars, retreats and conferences over the past years. Thanks to the members of **Anna Färnert´s**, **Anders Björkman´s**, **Göte Swedberg´s**, **Marita Troye-Blomberg´s** and **Klavs Berzons** groups. Also thanks to present and past board members of SMN: **Isabel Veiga**, **Pablo Giusti**, **Irina Jovel-Dalamu**, **Ulrika Morris**, **Klara Lundblom** and **Ioana Bujila**.

The service, IT and administrate units at MTC. The coordinators **Helene Stambeck** and **Anita Wallentin** for all the administrative help. **Birgitta Wester** for the help with FACS at MTC. **Greger, Sandór, Tage** and **Torbjörn** for all your help over the years! **Susanne Larsen** for your great work. Big thanks to **Milina, Rehijana** och **Jelena**.

My wonderful friends far away; to dear **Viviane**, thank you for always welcoming me to your home when I came to visit and for being supportive friend over all these years! To **Samiya**, **Waheed, Olivia, Samira** and **Fathiya** for all the wonderful memories and great dinners! To miss giggles, **Meesha** thank you for everything. You will always be remembered.

Till de absolut finaste tjejkompisar man kan önska sig, **Khadra Sucad** och **Amal!** Ni har varit med mig genom detta och så mycket mer tidigare. Ni vet hur mycket vårt vänskap betyder för mig. Tack så mycket för all ert stöd, kärlek, uppmuntran och alla härliga minnen genom dessa år. TACK SÅ MYCKET för att ni stod ut med mig de senaste åren (speciellt sista månaderna), och för att ni fanns där när jag behövde er!

Sist men inte minst min stora och härliga familj som har varit min fastpunkt hela mitt liv! Mina underbara systrar och bröder, **Zahra**, **Muna**, **Nimco**, **Liban** och **Gulaid**, och min moster **Sucad**. Ert kärlek, stöd, optimism och uppmuntran de senaste åren värderar jag otroligt högt. Lovar att svara på telefonen fr o m nu ;). Stort tack och jag älskar er så mycket!

To my wonderful parents**, Ahmed** and **Asha,** whom I have dedicated this work to.;
Waxan u soo jeedinayaa waalidkay ***Ahmed*** *iyo* ***Asha****. Kuwaas oo igu la dadaalay shaqadan. Waxan ugu mahad naqaayo jacayl kooda, dhiirigalintooda taageeradooda iyo wajihitan kooda sanadadan oo dhan. Waxan idiinku mahad naqayaa aamin sanidiina iyo ducadiinaba. Ana aan mar walba u cararaayey shaqo adag. una samaynayey marwalba wax run ahaantii aad uu sharaf badan ama wanaagsan. Is dhul dhigaaya kuna faanayaa in aan leeyahay laba waalid oo idin koo kala ah.*
Aad baan idiin jeclahay.
Mahadsanidiin!

7 REFERENCES

Abdel-Latif, M.S., Dietz, K., Issifou, S., Kremsner, P.G., and Klinkert, M.Q. (2003). Antibodies to Plasmodium falciparum rifin proteins are associated with rapid parasite clearance and asymptomatic infections. Infection and Immunity *71*, 6229-6233.

Abdel-Latif, M.S., Khattab, A., Lindenthal, C., Kremsner, P.G., and Klinkert, M.Q. (2002). Recognition of variant Rifin antigens by human antibodies induced during natural Plasmodium falciparum infections. Infection and Immunity *70*, 7013-7021.

Adams, J.H., Sim, B.K., Dolan, S.A., Fang, X., Kaslow, D.C., and Miller, L.H. (1992). A family of erythrocyte binding proteins of malaria parasites. P Natl Acad Sci USA *89*, 7085-7089.

Adda, C.G., Murphy, V.J., Sunde, M., Waddington, L.J., Schloegel, J., Talbo, G.H., Vingas, K., Kienzle, V., Masciantonio, R., Howlett, G.J.*, et al.* (2009). Plasmodium falciparum merozoite surface protein 2 is unstructured and forms amyloid-like fibrils. Mol Biochem Parasit *166*, 159-171.

Aide, P., Aponte, J.J., Renom, M., Nhampossa, T., Sacarlal, J., Mandomando, I., Bassat, Q., Manaca, M.N., Leach, A., Lievens, M.*, et al.* (2010). Safety, immunogenicity and duration of protection of the RTS,S/AS02(D) malaria vaccine: one year follow-up of a randomized controlled phase I/IIb trial. PLoS One *5*, e13838.

Aidoo, M., Terlouw, D.J., Kolczak, M.S., McElroy, P.D., ter Kuile, F.O., Kariuki, S., Nahlen, B.L., Lal, A.A., and Udhayakumar, V. (2002). Protective effects of the sickle cell gene against malaria morbidity and mortality. Lancet *359*, 1311-1312.

Aikawa, M. (1988a). Human cerebral malaria. The American journal of tropical medicine and hygiene *39*, 3-10.

Aikawa, M. (1988b). Morphological changes in erythrocytes induced by malarial parasites. Biol Cell *64*, 173-181.

Aikawa, M., Cochrane, A.H., Nussenzweig, R.S., and Rabbege, J. (1979). Freeze-fracture study of malaria sporozoites: antibody-induced changes of the pellicular membrane. The Journal of protozoology *26*, 273-279.

Aikawa, M., and Miller, L.H. (1983). Structural alteration of the erythrocyte membrane during malarial parasite invasion and intraerythrocytic development. Ciba Foundation symposium *94*, 45-63.

Aikawa, M., Miller, L.H., Johnson, J., and Rabbege, J. (1978). Erythrocyte entry by malarial parasites. A moving junction between erythrocyte and parasite. The Journal of cell biology *77*, 72-82.

Akpogheneta, O.J., Duah, N.O., Tetteh, K.K., Dunyo, S., Lanar, D.E., Pinder, M., and Conway, D.J. (2008). Duration of naturally acquired antibody responses to blood-stage Plasmodium falciparum is age dependent and antigen specific. Infection and Immunity *76*, 1748-1755.

Akpogheneta, O.J., Dunyo, S., Pinder, M., and Conway, D.J. (2010). Boosting antibody responses to Plasmodium falciparum merozoite antigens in children with highly seasonal exposure to infection. Parasite immunology *32*, 296-304.

al-Yaman, F., Awburn, M.M., and Clark, I.A. (1997). Serum creatinine levels and reactive nitrogen intermediates in children with cerebral malaria in Papua New Guinea. T Roy Soc Trop Med H *91*, 303-305.

al-Yaman, F., Genton, B., Anders, R., Taraika, J., Ginny, M., Mellor, S., and Alpers, M.P. (1995). Assessment of the role of the humoral response to Plasmodium falciparum MSP2 compared to RESA and SPf66 in protecting Papua New Guinean children from clinical malaria. Parasite immunology *17*, 493-501.

Alano, P., and Carter, R. (1990). Sexual differentiation in malaria parasites. Annual review of microbiology *44*, 429-449.

Allen, S.J., Bennett, S., Riley, E.M., Rowe, P.A., Jakobsen, P.H., O'Donnell, A., and Greenwood, B.M. (1992). Morbidity from malaria and immune responses to defined Plasmodium falciparum antigens in children with sickle cell trait in The Gambia. T Roy Soc Trop Med H *86*, 494-498.

Aly, A.S., and Matuschewski, K. (2005). A malarial cysteine protease is necessary for Plasmodium sporozoite egress from oocysts. The Journal of experimental medicine *202*, 225-230.

Amino, R., Giovannini, D., Thiberge, S., Gueirard, P., Boisson, B., Dubremetz, J.F., Prevost, M.C., Ishino, T., Yuda, M., and Menard, R. (2008). Host cell traversal is important for progression of the malaria parasite through the dermis to the liver. Cell host & microbe *3*, 88-96.

Amino, R., Thiberge, S., Martin, B., Celli, S., Shorte, S., Frischknecht, F., and Menard, R. (2006). Quantitative imaging of Plasmodium transmission from mosquito to mammal. Nature Medicine *12*, 220-224.

Anand, A.C., and Puri, P. (2005). Jaundice in malaria. Journal of gastroenterology and hepatology *20*, 1322-1332.

Anders, R.F., Adda, C.G., Foley, M., and Norton, R.S. (2010). Recombinant protein vaccines against the asexual blood stages of Plasmodium falciparum. Human vaccines *6*, 39-53.

Anstey, N.M., Douglas, N.M., Poespoprodjo, J.R., and Price, R.N. (2012). Plasmodium vivax: clinical spectrum, risk factors and pathogenesis. Advances in parasitology *80*, 151-201.

Arias-Bouda, L.M., Kuijper, S., Van der Werf, A., Nguyen, L.N., Jansen, H.M., and Kolk, A.H. (2003). Changes in avidity and level of immunoglobulin G antibodies to Mycobacterium tuberculosis in sera of patients undergoing treatment for pulmonary tuberculosis. Clin Diagn Lab Immun *10*, 702-709.

Arnot, D. (1998). Unstable malaria in Sudan: the influence of the dry season. Clone multiplicity of Plasmodium falciparum infections in individuals exposed to variable levels of disease transmission. T Roy Soc Trop Med H *92*, 580-585.

Atkinson, C.T., and Aikawa, M. (1990). Ultrastructure of malaria-infected erythrocytes. Blood Cells *16*, 351-368.

Aucan, C., Traore, Y., Tall, F., Nacro, B., Traore-Leroux, T., Fumoux, F., and Rihet, P. (2000). High immunoglobulin G2 (IgG2) and low IgG4 levels are associated with human resistance to Plasmodium falciparum malaria. Infection and Immunity *68*, 1252-1258.

Baer, K., Klotz, C., Kappe, S.H., Schnieder, T., and Frevert, U. (2007a). Release of hepatic Plasmodium yoelii merozoites into the pulmonary microvasculature. PLoS pathogens *3*, e171.

Baer, K., Roosevelt, M., Clarkson, A.B., Jr., van Rooijen, N., Schnieder, T., and Frevert, U. (2007b). Kupffer cells are obligatory for Plasmodium yoelii sporozoite infection of the liver. Cellular microbiology *9*, 397-412.

Bannister, L.H., and Dluzewski, A.R. (1990). The ultrastructure of red cell invasion in malaria infections: a review. Blood Cells *16*, 257-292; discussion 293-257.

Bannister, L.H., Hopkins, J.M., Margos, G., Dluzewski, A.R., and Mitchell, G.H. (2004). Three-dimensional ultrastructure of the ring stage of Plasmodium falciparum: evidence for export pathways. Microscopy and microanalysis : the official journal of Microscopy Society of America, Microbeam Analysis Society, Microscopical Society of Canada *10*, 551-562.

Baratin, M., Roetynck, S., Pouvelle, B., Lemmers, C., Viebig, N.K., Johansson, S., Bierling, P., Scherf, A., Gysin, J., Vivier, E., *et al.* (2007). Dissection of the role of PfEMP1 and ICAM-1 in the sensing of Plasmodium-falciparum-infected erythrocytes by natural killer cells. PLoS One *2*, e228.

Barcus, M.J., Basri, H., Picarima, H., Manyakori, C., Sekartuti, Elyazar, I., Bangs, M.J., Maguire, J.D., and Baird, J.K. (2007). Demographic risk factors for severe and fatal vivax and falciparum malaria among hospital admissions in northeastern Indonesian Papua. The American journal of tropical medicine and hygiene *77*, 984-991.

Barragan, A., Fernandez, V., Chen, Q., von Euler, A., Wahlgren, M., and Spillmann, D. (2000 a). The duffy-binding-like domain 1 of Plasmodium falciparum erythrocyte membrane protein 1 (PfEMP1) is a heparan sulfate ligand that requires 12 mers for binding. Blood *95*, 3594-3599.

Barragan, A., Kremsner, P.G., Wahlgren, M., and Carlson, J. (2000 b). Blood group A antigen is a coreceptor in Plasmodium falciparum rosetting. Infection and Immunity *68*, 2971-2975.

Barry, A.E., Schultz, L., Buckee, C.O., and Reeder, J.C. (2009). Contrasting population structures of the genes encoding ten leading vaccine-candidate antigens of the human malaria parasite, Plasmodium falciparum. PLoS One *4*, e8497.

Baruch, D.I., Gormely, J.A., Ma, C., Howard, R.J., and Pasloske, B.L. (1996). Plasmodium falciparum erythrocyte membrane protein 1 is a parasitized erythrocyte receptor for adherence to CD36, thrombospondin, and intercellular adhesion molecule 1. P Natl Acad Sci USA *93*, 3497-3502.

Baum, J., Maier, A.G., Good, R.T., Simpson, K.M., and Cowman, A.F. (2005). Invasion by P. falciparum merozoites suggests a hierarchy of molecular interactions. PLoS pathogens *1*, e37.

Beeson, J.G., Brown, G.V., Molyneux, M.E., Mhango, C., Dzinjalamala, F., and Rogerson, S.J. (1999). Plasmodium falciparum isolates from infected pregnant women and children are associated with distinct adhesive and antigenic properties. The Journal of infectious diseases *180*, 464-472.

Beeson, J.G., Rogerson, S.J., Cooke, B.M., Reeder, J.C., Chai, W., Lawson, A.M., Molyneux, M.E., and Brown, G.V. (2000). Adhesion of Plasmodium falciparum-infected erythrocytes to hyaluronic acid in placental malaria. Nature Medicine *6*, 86-90.

Bir, N., Yazdani, S.S., Avril, M., Layez, C., Gysin, J., and Chitnis, C.E. (2006). Immunogenicity of Duffy binding-like domains that bind chondroitin sulfate A and protection against pregnancy-associated malaria. Infection and Immunity *74*, 5955-5963.

Blackman, M.J., Heidrich, H.G., Donachie, S., McBride, J.S., and Holder, A.A. (1990). A single fragment of a malaria merozoite surface protein remains on the parasite during red cell invasion and is the target of invasion-inhibiting antibodies. The Journal of experimental medicine *172*, 379-382.

Blackman, M.J., and Holder, A.A. (1992). Secondary processing of the Plasmodium falciparum merozoite surface protein-1 (MSP1) by a calcium-dependent membrane-bound serine protease: shedding of MSP133 as a noncovalently associated complex with other fragments of the MSP1. Mol Biochem Parasit *50*, 307-315.

Blair, P.L., Kappe, S.H., Maciel, J.E., Balu, B., and Adams, J.H. (2002). Plasmodium falciparum MAEBL is a unique member of the ebl family. Mol Biochem Parasit *122*, 35-44.

Blythe, J.E., Yam, X.Y., Kuss, C., Bozdech, Z., Holder, A.A., Marsh, K., Langhorne, J., and Preiser, P.R. (2008). Plasmodium falciparum STEVOR proteins are highly expressed in patient isolates and located in the surface membranes of infected red blood cells and the apical tips of merozoites. Infection and Immunity *76*, 3329-3336.

Boddey, J.A., and Cowman, A.F. (2013). Plasmodium nesting: remaking the erythrocyte from the inside out. Annual review of microbiology *67*, 243-269.

Bondi, F.S. (1992). The incidence and outcome of neurological abnormalities in childhood cerebral malaria: a long-term follow-up of 62 survivors. T Roy Soc Trop Med H *86*, 17-19.

Bouharoun-Tayoun, H., Attanath, P., Sabchareon, A., Chongsuphajaisiddhi, T., and Druilhe, P. (1990). Antibodies that protect humans against Plasmodium falciparum blood stages do not on their own inhibit parasite growth and invasion in vitro, but act in cooperation with monocytes. The Journal of experimental medicine *172*, 1633-1641.

Bouharoun-Tayoun, H., and Druilhe, P. (1992). Plasmodium falciparum malaria: evidence for an isotype imbalance which may be responsible for delayed acquisition of protective immunity. Infection and Immunity *60*, 1473-1481.

Bouharoun-Tayoun, H., Oeuvray, C., Lunel, F., and Druilhe, P. (1995). Mechanisms underlying the monocyte-mediated antibody-dependent killing of Plasmodium falciparum asexual blood stages. The Journal of experimental medicine *182*, 409-418.

Boyle, M.J., Langer, C., Chan, J.A., Hodder, A.N., Coppel, R.L., Anders, R.F., and Beeson, J.G. (2013). Sequential processing of merozoite surface proteins during and after erythrocyte invasion by Plasmodium falciparum. Infection and Immunity.

Boyle, M.J., Richards, J.S., Gilson, P.R., Chai, W., and Beeson, J.G. (2010). Interactions with heparin-like molecules during erythrocyte invasion by Plasmodium falciparum merozoites. Blood *115*, 4559-4568.

Branch, O.H., Udhayakumar, V., Hightower, A.W., Oloo, A.J., Hawley, W.A., Nahlen, B.L., Bloland, P.B., Kaslow, D.C., and Lal, A.A. (1998). A longitudinal investigation of IgG and IgM antibody responses to the merozoite surface protein-1 19-kiloDalton domain of Plasmodium falciparum in pregnant women and infants: associations with febrile illness, parasitemia, and anemia. The American journal of tropical medicine and hygiene *58*, 211-219.

Bruce, M.C., Alano, P., Duthie, S., and Carter, R. (1990). Commitment of the malaria parasite Plasmodium falciparum to sexual and asexual development. Parasitology *100 Pt 2*, 191-200.

Bull, P.C., Berriman, M., Kyes, S., Quail, M.A., Hall, N., Kortok, M.M., Marsh, K., and Newbold, C.I. (2005). Plasmodium falciparum variant surface antigen expression patterns during malaria. PLoS pathogens *1*, e26.

Bull, P.C., Lowe, B.S., Kortok, M., and Marsh, K. (1999). Antibody recognition of Plasmodium falciparum erythrocyte surface antigens in Kenya: evidence for rare and prevalent variants. Infection and Immunity *67*, 733-739.

Bunn, H.F. (2013). The triumph of good over evil: protection by the sickle gene against malaria. Blood *121*, 20-25.

Buscaglia, C.A., Hol, W.G., Nussenzweig, V., and Cardozo, T. (2007). Modeling the interaction between aldolase and the thrombospondin-related anonymous protein, a key connection of the malaria parasite invasion machinery. Proteins *66*, 528-537.

Camus, D., and Hadley, T.J. (1985). A Plasmodium falciparum antigen that binds to host erythrocytes and merozoites. Science *230*, 553-556.

Carlson, J., Ekre, H.P., Helmby, H., Gysin, J., Greenwood, B.M., and Wahlgren, M. (1992). Disruption of Plasmodium falciparum erythrocyte rosettes by standard heparin and heparin devoid of anticoagulant activity. The American journal of tropical medicine and hygiene *46*, 595-602.

Carlson, J., Helmby, H., Hill, A.V., Brewster, D., Greenwood, B.M., and Wahlgren, M. (1990). Human cerebral malaria: association with erythrocyte rosetting and lack of anti-rosetting antibodies. Lancet *336*, 1457-1460.

Carlson, J., Nash, G.B., Gabutti, V., al-Yaman, F., and Wahlgren, M. (1994). Natural protection against severe Plasmodium falciparum malaria due to impaired rosette formation. Blood *84*, 3909-3914.

Carlson, J., and Wahlgren, M. (1992). Plasmodium falciparum erythrocyte rosetting is mediated by promiscuous lectin-like interactions. The Journal of experimental medicine *176*, 1311-1317.

Chattopadhyay, R., Taneja, T., Chakrabarti, K., Pillai, C.R., and Chitnis, C.E. (2004). Molecular analysis of the cytoadherence phenotype of a Plasmodium falciparum field isolate that binds intercellular adhesion molecule-1. Mol Biochem Parasit *133*, 255-265.

Chaves, L.F., Taleo, G., Kalkoa, M., and Kaneko, A. (2011). Spleen rates in children: an old and new surveillance tool for malaria elimination initiatives in island settings. T Roy Soc Trop Med H *105*, 226-231.

Chen, Q., Barragan, A., Fernandez, V., Sundstrom, A., Schlichtherle, M., Sahlen, A., Carlson, J., Datta, S., and Wahlgren, M. (1998). Identification of Plasmodium falciparum erythrocyte membrane protein 1 (PfEMP1) as the rosetting ligand of the malaria parasite P. falciparum. The Journal of experimental medicine *187*, 15-23.

Cheng, Q., Cloonan, N., Fischer, K., Thompson, J., Waine, G., Lanzer, M., and Saul, A. (1998). stevor and rif are Plasmodium falciparum multicopy gene families which potentially encode variant antigens. Mol Biochem Parasit *97*, 161-176.

Cheng, Q., and Saul, A. (1994). Sequence analysis of the apical membrane antigen I (AMA-1) of Plasmodium vivax. Mol Biochem Parasit *65*, 183-187.

Cockburn, I.A., Mackinnon, M.J., O'Donnell, A., Allen, S.J., Moulds, J.M., Baisor, M., Bockarie, M., Reeder, J.C., and Rowe, J.A. (2004). A human complement receptor 1 polymorphism that reduces Plasmodium falciparum rosetting confers protection against severe malaria. P Natl Acad Sci USA *101*, 272-277.

Cohen, S., Butcher, G.A., and Crandall, R.B. (1969). Action of malarial antibody in vitro. Nature *223*, 368-371.

Cohen, S., Mc, G.I., and Carrington, S. (1961). Gamma-globulin and acquired immunity to human malaria. Nature *192*, 733-737.

Collins, C.R., Withers-Martinez, C., Hackett, F., and Blackman, M.J. (2009). An inhibitory antibody blocks interactions between components of the malarial invasion machinery. PLoS pathogens *5*, e1000273.

Cooke, B.M., Berendt, A.R., Craig, A.G., MacGregor, J., Newbold, C.I., and Nash, G.B. (1994). Rolling and stationary cytoadhesion of red blood cells parasitized by Plasmodium falciparum: separate roles for ICAM-1, CD36 and thrombospondin. Brit J Haematol *87*, 162-170.

Cooke, B.M., Buckingham, D.W., Glenister, F.K., Fernandez, K.M., Bannister, L.H., Marti, M., Mohandas, N., and Coppel, R.L. (2006). A Maurer's cleft-associated protein is essential for expression of the major malaria virulence antigen on the surface of infected red blood cells. The Journal of cell biology *172*, 899-908.

Cooke, B.M., Lingelbach, K., Bannister, L.H., and Tilley, L. (2004). Protein trafficking in Plasmodium falciparum-infected red blood cells. Trends in parasitology *20*, 581-589.

Corran, P.H., O'Donnell, R.A., Todd, J., Uthaipibull, C., Holder, A.A., Crabb, B.S., and Riley, E.M. (2004). The fine specificity, but not the invasion inhibitory activity, of 19-kilodalton merozoite surface protein 1-specific antibodies is associated with resistance to malarial parasitemia in a cross-sectional survey in The Gambia. Infection and Immunity *72*, 6185-6189.

Cortes, A., Mellombo, M., Masciantonio, R., Murphy, V.J., Reeder, J.C., and Anders, R.F. (2005). Allele specificity of naturally acquired antibody responses against Plasmodium falciparum apical membrane antigen 1. Infection and Immunity *73*, 422-430.

Cortes, A., Mellombo, M., Mueller, I., Benet, A., Reeder, J.C., and Anders, R.F. (2003). Geographical structure of diversity and differences between symptomatic and asymptomatic infections for Plasmodium falciparum vaccine candidate AMA1. Infection and Immunity *71*, 1416-1426.

Courtin, D., Oesterholt, M., Huismans, H., Kusi, K., Milet, J., Badaut, C., Gaye, O., Roeffen, W., Remarque, E.J., Sauerwein, R.*, et al.* (2009). The quantity and quality of African children's IgG responses to merozoite surface antigens reflect protection against Plasmodium falciparum malaria. PLoS One *4*, e7590.

Cowman, A.F., and Crabb, B.S. (2006). Invasion of red blood cells by malaria parasites. Cell *124*, 755-766.

Crabb, B.S., Cooke, B.M., Reeder, J.C., Waller, R.F., Caruana, S.R., Davern, K.M., Wickham, M.E., Brown, G.V., Coppel, R.L., and Cowman, A.F. (1997). Targeted gene disruption shows that knobs enable malaria-infected red cells to cytoadhere under physiological shear stress. Cell *89*, 287-296.

Crawley, J., Smith, S., Kirkham, F., Muthinji, P., Waruiru, C., and Marsh, K. (1996). Seizures and status epilepticus in childhood cerebral malaria. QJM : monthly journal of the Association of Physicians *89*, 591-597.

Crawley, J., Smith, S., Muthinji, P., Marsh, K., and Kirkham, F. (2001). Electroencephalographic and clinical features of cerebral malaria. Archives of disease in childhood *84*, 247-253.

Creasey, A.M., Staalsoe, T., Raza, A., Arnot, D.E., and Rowe, J.A. (2003). Nonspecific immunoglobulin M binding and chondroitin sulfate A binding are linked phenotypes of Plasmodium falciparum isolates implicated in malaria during pregnancy. Infection and Immunity *71*, 4767-4771.

Crompton, P.D., Miura, K., Traore, B., Kayentao, K., Ongoiba, A., Weiss, G., Doumbo, S., Doumtabe, D., Kone, Y., Huang, C.Y.*, et al.* (2010). In vitro growth-inhibitory activity and malaria risk in a cohort study in mali. Infection and Immunity *78*, 737-745.

Crompton, P.D., Traore, B., Kayentao, K., Doumbo, S., Ongoiba, A., Diakite, S.A., Krause, M.A., Doumtabe, D., Kone, Y., Weiss, G.*, et al.* (2008). Sickle cell trait is associated with a delayed onset of malaria: implications for time-to-event analysis in clinical studies of malaria. The Journal of infectious diseases *198*, 1265-1275.

Crosnier, C., Bustamante, L.Y., Bartholdson, S.J., Bei, A.K., Theron, M., Uchikawa, M., Mboup, S., Ndir, O., Kwiatkowski, D.P., Duraisingh, M.T.*, et al.* (2011). Basigin is a

receptor essential for erythrocyte invasion by Plasmodium falciparum. Nature *480*, 534-537.

Culvenor, J.G., Langford, C.J., Crewther, P.E., Saint, R.B., Coppel, R.L., Kemp, D.J., Anders, R.F., and Brown, G.V. (1987). Plasmodium falciparum: identification and localization of a knob protein antigen expressed by a cDNA clone. Experimental parasitology *63*, 58-67.

Czajkowsky, D.M., Salanti, A., Ditlev, S.B., Shao, Z., Ghumra, A., Rowe, J.A., and Pleass, R.J. (2010). IgM, Fc mu Rs, and malarial immune evasion. Journal of immunology *184*, 4597-4603.

Czajkowsky, D.M., and Shao, Z. (2009). The human IgM pentamer is a mushroom-shaped molecule with a flexural bias. P Natl Acad Sci USA *106*, 14960-14965.

Deans, J.A., Knight, A.M., Jean, W.C., Waters, A.P., Cohen, S., and Mitchell, G.H. (1988). Vaccination trials in rhesus monkeys with a minor, invariant, Plasmodium knowlesi 66 kD merozoite antigen. Parasite immunology *10*, 535-552.

Del Portillo, H.A., Ferrer, M., Brugat, T., Martin-Jaular, L., Langhorne, J., and Lacerda, M.V. (2012). The role of the spleen in malaria. Cellular microbiology *14*, 343-355.

Dent, A.E., Bergmann-Leitner, E.S., Wilson, D.W., Tisch, D.J., Kimmel, R., Vulule, J., Sumba, P.O., Beeson, J.G., Angov, E., Moormann, A.M.*, et al.* (2008). Antibody-mediated growth inhibition of Plasmodium falciparum: relationship to age and protection from parasitemia in Kenyan children and adults. PLoS One *3*, e3557.

Desai, S.A., Krogstad, D.J., and McCleskey, E.W. (1993). A nutrient-permeable channel on the intraerythrocytic malaria parasite. Nature *362*, 643-646.

DeSimone, T.M., Bei, A.K., Jennings, C.V., and Duraisingh, M.T. (2009). Genetic analysis of the cytoplasmic domain of the PfRh2b merozoite invasion protein of Plasmodium falciparum. International journal for parasitology *39*, 399-405.

Dicko, A., Sagara, I., Ellis, R.D., Miura, K., Guindo, O., Kamate, B., Sogoba, M., Niambele, M.B., Sissoko, M., Baby, M.*, et al.* (2008). Phase 1 study of a combination AMA1 blood stage malaria vaccine in Malian children. PLoS One *3*, e1563.

Dinglasan, R.R., Armistead, J.S., Nyland, J.F., Jiang, X., and Mao, H.Q. (2013). Single-dose microparticle delivery of a malaria transmission-blocking vaccine elicits a long-lasting functional antibody response. Current molecular medicine *13*, 479-487.

Dluzewski, A.R., Ling, I.T., Hopkins, J.M., Grainger, M., Margos, G., Mitchell, G.H., Holder, A.A., and Bannister, L.H. (2008). Formation of the food vacuole in Plasmodium falciparum: a potential role for the 19 kDa fragment of merozoite surface protein 1 (MSP1(19)). PLoS One *3*, e3085.

Dobano, C., Quelhas, D., Quinto, L., Puyol, L., Serra-Casas, E., Mayor, A., Nhampossa, T., Macete, E., Aide, P., Mandomando, I.*, et al.* (2012). Age-dependent IgG subclass responses to Plasmodium falciparum EBA-175 are differentially associated with incidence of malaria in Mozambican children. Clinical and vaccine immunology : CVI *19*, 157-166.

Dolan, S.A., Miller, L.H., and Wellems, T.E. (1990). Evidence for a switching mechanism in the invasion of erythrocytes by Plasmodium falciparum. The Journal of clinical investigation *86*, 618-624.

Dondorp, A.M., Nosten, F., Yi, P., Das, D., Phyo, A.P., Tarning, J., Lwin, K.M., Ariey, F., Hanpithakpong, W., Lee, S.J.*, et al.* (2009). Artemisinin resistance in Plasmodium falciparum malaria. The New England journal of medicine *361*, 455-467.

Doolan, D.L., Dobano, C., and Baird, J.K. (2009). Acquired immunity to malaria. Clinical microbiology reviews *22*, 13-36, Table of Contents.

Duarte, M.I., Corbett, C.E., Boulos, M., and Amato Neto, V. (1985). Ultrastructure of the lung in falciparum malaria. The American journal of tropical medicine and hygiene *34*, 31-35.

Duffy, P.E., and Fried, M. (2005). Malaria in the pregnant woman. Current topics in microbiology and immunology *295*, 169-200.

Duraisingh, M.T., Maier, A.G., Triglia, T., and Cowman, A.F. (2003a). Erythrocyte-binding antigen 175 mediates invasion in Plasmodium falciparum utilizing sialic acid-dependent and -independent pathways. P Natl Acad Sci USA *100*, 4796-4801.

Duraisingh, M.T., Triglia, T., Ralph, S.A., Rayner, J.C., Barnwell, J.W., McFadden, G.I., and Cowman, A.F. (2003b). Phenotypic variation of Plasmodium falciparum merozoite proteins directs receptor targeting for invasion of human erythrocytes. The EMBO journal *22*, 1047-1057.

Dutta, S., Kaushal, D.C., Ware, L.A., Puri, S.K., Kaushal, N.A., Narula, A., Upadhyaya, D.S., and Lanar, D.E. (2005). Merozoite surface protein 1 of Plasmodium vivax induces a protective response against Plasmodium cynomolgi challenge in rhesus monkeys. Infection and Immunity *73*, 5936-5944.

Dutta, S., Lalitha, P.V., Ware, L.A., Barbosa, A., Moch, J.K., Vassell, M.A., Fileta, B.B., Kitov, S., Kolodny, N., Heppner, D.G.*, et al.* (2002). Purification, characterization, and immunogenicity of the refolded ectodomain of the Plasmodium falciparum apical membrane antigen 1 expressed in Escherichia coli. Infection and Immunity *70*, 3101-3110.

Dutta, S., Malhotra, P., and Chauhan, V.S. (1995). Sequence analysis of apical membrane antigen 1 (AMA-1) of Plasmodium cynomolgi bastianelli. Mol Biochem Parasit *73*, 267-270.

Dvorak, J.A., Miller, L.H., Whitehouse, W.C., and Shiroishi, T. (1975). Invasion of erythrocytes by malaria merozoites. Science *187*, 748-750.

Dvorin, J.D., Bei, A.K., Coleman, B.I., and Duraisingh, M.T. (2010a). Functional diversification between two related Plasmodium falciparum merozoite invasion ligands is determined by changes in the cytoplasmic domain. Molecular microbiology *75*, 990-1006.

Dvorin, J.D., Martyn, D.C., Patel, S.D., Grimley, J.S., Collins, C.R., Hopp, C.S., Bright, A.T., Westenberger, S., Winzeler, E., Blackman, M.J.*, et al.* (2010b). A plant-like kinase in Plasmodium falciparum regulates parasite egress from erythrocytes. Science *328*, 910-912.

Dzikowski, R., Frank, M., and Deitsch, K. (2006). Mutually exclusive expression of virulence genes by malaria parasites is regulated independently of antigen production. PLoS pathogens *2*, e22.

Egan, A.F., Morris, J., Barnish, G., Allen, S., Greenwood, B.M., Kaslow, D.C., Holder, A.A., and Riley, E.M. (1996). Clinical immunity to Plasmodium falciparum malaria is associated with serum antibodies to the 19-kDa C-terminal fragment of the merozoite surface antigen, PfMSP-1. The Journal of infectious diseases *173*, 765-769.

El Sahly, H.M., Patel, S.M., Atmar, R.L., Lanford, T.A., Dube, T., Thompson, D., Sim, B.K., Long, C., and Keitel, W.A. (2010). Safety and immunogenicity of a recombinant nonglycosylated erythrocyte binding antigen 175 Region II malaria vaccine in healthy adults living in an area where malaria is not endemic. Clinical and vaccine immunology : CVI *17*, 1552-1559.

Elmendorf, H.G., and Haldar, K. (1994). Plasmodium falciparum exports the Golgi marker sphingomyelin synthase into a tubovesicular network in the cytoplasm of mature erythrocytes. The Journal of cell biology *124*, 449-462.

English, M., Waruiru, C., Amukoye, E., Murphy, S., Crawley, J., Mwangi, I., Peshu, N., and Marsh, K. (1996). Deep breathing in children with severe malaria: indicator of metabolic acidosis and poor outcome. The American journal of tropical medicine and hygiene *55*, 521-524.

Evans, K.J., Hansen, D.S., van Rooijen, N., Buckingham, L.A., and Schofield, L. (2006). Severe malarial anemia of low parasite burden in rodent models results from accelerated clearance of uninfected erythrocytes. Blood *107*, 1192-1199.

Fairhurst, R.M., Baruch, D.I., Brittain, N.J., Ostera, G.R., Wallach, J.S., Hoang, H.L., Hayton, K., Guindo, A., Makobongo, M.O., Schwartz, O.M.*, et al.* (2005). Abnormal display of PfEMP-1 on erythrocytes carrying haemoglobin C may protect against malaria. Nature *435*, 1117-1121.

Faiz, M.A., Rahman, M.R., Hossain, M.A., and Rashid, H.A. (1998). Cerebral malaria--a study of 104 cases. Bangladesh Med Res Counc Bull *24*, 35-42.

Farnert, A., Williams, T.N., Mwangi, T.W., Ehlin, A., Fegan, G., Macharia, A., Lowe, B.S., Montgomery, S.M., and Marsh, K. (2009). Transmission-dependent tolerance to multiclonal Plasmodium falciparum infection. The Journal of infectious diseases *200*, 1166-1175.

Felger, I., Tavul, L., Kabintik, S., Marshall, V., Genton, B., Alpers, M., and Beck, H.P. (1994). Plasmodium falciparum: extensive polymorphism in merozoite surface antigen 2 alleles in an area with endemic malaria in Papua New Guinea. Experimental parasitology *79*, 106-116.

Fenton, B., Clark, J.T., Khan, C.M., Robinson, J.V., Walliker, D., Ridley, R., Scaife, J.G., and McBride, J.S. (1991). Structural and antigenic polymorphism of the 35- to 48-kilodalton

merozoite surface antigen (MSA-2) of the malaria parasite Plasmodium falciparum. Molecular and Cellular Biology *11*, 963-971.

Ferreira, M.U., Ribeiro, W.L., Tonon, A.P., Kawamoto, F., and Rich, S.M. (2003). Sequence diversity and evolution of the malaria vaccine candidate merozoite surface protein-1 (MSP-1) of Plasmodium falciparum. Gene *304*, 65-75.

Fleming, A.F., Storey, J., Molineaux, L., Iroko, E.A., and Attai, E.D. (1979). Abnormal haemoglobins in the Sudan savanna of Nigeria. I. Prevalence of haemoglobins and relationships between sickle cell trait, malaria and survival. Ann Trop Med Parasit *73*, 161-172.

Flick, K., and Chen, Q. (2004). var genes, PfEMP1 and the human host. Mol Biochem Parasit *134*, 3-9.

Flick, K., Scholander, C., Chen, Q., Fernandez, V., Pouvelle, B., Gysin, J., and Wahlgren, M. (2001). Role of nonimmune IgG bound to PfEMP1 in placental malaria. Science *293*, 2098-2100.

Florens, L., Washburn, M.P., Raine, J.D., Anthony, R.M., Grainger, M., Haynes, J.D., Moch, J.K., Muster, N., Sacci, J.B., Tabb, D.L.*, et al.* (2002). A proteomic view of the Plasmodium falciparum life cycle. Nature *419*, 520-526.

Flueck, C., Frank, G., Smith, T., Jafarshad, A., Nebie, I., Sirima, S.B., Olugbile, S., Alonso, P., Tanner, M., Druilhe, P.*, et al.* (2009). Evaluation of two long synthetic merozoite surface protein 2 peptides as malaria vaccine candidates. Vaccine *27*, 2653-2661.

Ford, L., Lobo, C.A., Rodriguez, M., Zalis, M.G., Machado, R.L., Rossit, A.R., Cavasini, C.E., Couto, A.A., Enyong, P.A., and Lustigman, S. (2007). Differential antibody responses to Plasmodium falciparum invasion ligand proteins in individuals living in malaria-endemic areas in Brazil and Cameroon. The American journal of tropical medicine and hygiene *77*, 977-983.

Fowkes, F.J., Richards, J.S., Simpson, J.A., and Beeson, J.G. (2010). The relationship between anti-merozoite antibodies and incidence of Plasmodium falciparum malaria: A systematic review and meta-analysis. PLoS medicine *7*, e1000218.

Fraser, T.S., Kappe, S.H., Narum, D.L., VanBuskirk, K.M., and Adams, J.H. (2001). Erythrocyte-binding activity of Plasmodium yoelii apical membrane antigen-1 expressed on the surface of transfected COS-7 cells. Mol Biochem Parasit *117*, 49-59.

Freitas-Junior, L.H., Bottius, E., Pirrit, L.A., Deitsch, K.W., Scheidig, C., Guinet, F., Nehrbass, U., Wellems, T.E., and Scherf, A. (2000). Frequent ectopic recombination of virulence factor genes in telomeric chromosome clusters of P. falciparum. Nature *407*, 1018-1022.

Frevert, U., Engelmann, S., Zougbede, S., Stange, J., Ng, B., Matuschewski, K., Liebes, L., and Yee, H. (2005). Intravital observation of Plasmodium berghei sporozoite infection of the liver. PLoS biology *3*, e192.

Frevert, U., and Nardin, E. (2008). Cellular effector mechanisms against Plasmodium liver stages. Cellular microbiology *10*, 1956-1967.

Fried, M., and Duffy, P.E. (1996). Adherence of Plasmodium falciparum to chondroitin sulfate A in the human placenta. Science *272*, 1502-1504.

Fried, M., Nosten, F., Brockman, A., Brabin, B.J., and Duffy, P.E. (1998). Maternal antibodies block malaria. Nature *395*, 851-852.

Friedman, S., Back, B., Delivoria-Papadopoulos, M., Atwater, J., Asakura, T., and Schwartz, E. (1978). A simple test for detection of sickle hemoglobin in the neonatal period. American journal of clinical pathology *70*, 85-88.

Galinski, M.R., Medina, C.C., Ingravallo, P., and Barnwell, J.W. (1992). A reticulocyte-binding protein complex of Plasmodium vivax merozoites. Cell *69*, 1213-1226.

Gardner, M.J., Hall, N., Fung, E., White, O., Berriman, M., Hyman, R.W., Carlton, J.M., Pain, A., Nelson, K.E., Bowman, S.*, et al.* (2002). Genome sequence of the human malaria parasite Plasmodium falciparum. Nature *419*, 498-511.

Garraud, O., Mahanty, S., and Perraut, R. (2003). Malaria-specific antibody subclasses in immune individuals: a key source of information for vaccine design. Trends in immunology *24*, 30-35.

Gaur, D., and Chitnis, C.E. (2011). Molecular interactions and signaling mechanisms during erythrocyte invasion by malaria parasites. Current opinion in microbiology *14*, 422-428.

Gaur, D., Furuya, T., Mu, J., Jiang, L.B., Su, X.Z., and Miller, L.H. (2006). Upregulation of expression of the reticulocyte homology gene 4 in the Plasmodium falciparum clone Dd2 is associated with a switch in the erythrocyte invasion pathway. Mol Biochem Parasit *145*, 205-215.

Gaur, D., Storry, J.R., Reid, M.E., Barnwell, J.W., and Miller, L.H. (2003). Plasmodium falciparum is able to invade erythrocytes through a trypsin-resistant pathway independent of glycophorin B. Infection and Immunity *71*, 6742-6746.

Genton, B. (2008). Malaria vaccines: a toy for travelers or a tool for eradication? Expert review of vaccines *7*, 597-611.

Ghumra, A., Semblat, J.P., McIntosh, R.S., Raza, A., Rasmussen, I.B., Braathen, R., Johansen, F.E., Sandlie, I., Mongini, P.K., Rowe, J.A*., et al.* (2008). Identification of residues in the Cmu4 domain of polymeric IgM essential for interaction with Plasmodium falciparum erythrocyte membrane protein 1 (PfEMP1). Journal of immunology *181*, 1988-2000.

Gilberger, T.W., Thompson, J.K., Reed, M.B., Good, R.T., and Cowman, A.F. (2003). The cytoplasmic domain of the Plasmodium falciparum ligand EBA-175 is essential for invasion but not protein trafficking. The Journal of cell biology *162*, 317-327.

Gilson, P.R., and Crabb, B.S. (2009). Morphology and kinetics of the three distinct phases of red blood cell invasion by Plasmodium falciparum merozoites. International journal for parasitology *39*, 91-96.

Gilson, P.R., O'Donnell, R.A., Nebl, T., Sanders, P.R., Wickham, M.E., McElwain, T.F., de Koning-Ward, T.F., and Crabb, B.S. (2008). MSP1(19) miniproteins can serve as targets for invasion inhibitory antibodies in Plasmodium falciparum provided they contain the correct domains for cell surface trafficking. Molecular microbiology *68*, 124-138.

Glushakova, S., Yin, D., Li, T., and Zimmerberg, J. (2005). Membrane transformation during malaria parasite release from human red blood cells. Current biology : CB *15*, 1645-1650.

Goel, V.K., Li, X., Chen, H., Liu, S.C., Chishti, A.H., and Oh, S.S. (2003). Band 3 is a host receptor binding merozoite surface protein 1 during the Plasmodium falciparum invasion of erythrocytes. P Natl Acad Sci USA *100*, 5164-5169.

Goldberg, D.E., and Cowman, A.F. (2010). Moving in and renovating: exporting proteins from Plasmodium into host erythrocytes. Nature reviews Microbiology *8*, 617-621.

Gomez-Escobar, N., Amambua-Ngwa, A., Walther, M., Okebe, J., Ebonyi, A., and Conway, D.J. (2010). Erythrocyte invasion and merozoite ligand gene expression in severe and mild Plasmodium falciparum malaria. The Journal of infectious diseases *201*, 444-452.

Goodman, A.L., and Draper, S.J. (2010). Blood-stage malaria vaccines - recent progress and future challenges. Ann Trop Med Parasit *104*, 189-211.

Greenhouse, B., Ho, B., Hubbard, A., Njama-Meya, D., Narum, D.L., Lanar, D.E., Dutta, S., Rosenthal, P.J., Dorsey, G., and John, C.C. (2011). Antibodies to Plasmodium falciparum antigens predict a higher risk of malaria but protection from symptoms once parasitemic. The Journal of infectious diseases *204*, 19-26.

Gupta, S., Snow, R.W., Donnelly, C., and Newbold, C. (1999a). Acquired immunity and postnatal clinical protection in childhood cerebral malaria. Proceedings Biological sciences / The Royal Society *266*, 33-38.

Gupta, S., Snow, R.W., Donnelly, C.A., Marsh, K., and Newbold, C. (1999b). Immunity to non-cerebral severe malaria is acquired after one or two infections. Nature Medicine *5*, 340-343.

Haase, S., and de Koning-Ward, T.F. (2010). New insights into protein export in malaria parasites. Cellular microbiology *12*, 580-587.

Haeggstrom, M., Kironde, F., Berzins, K., Chen, Q., Wahlgren, M., and Fernandez, V. (2004). Common trafficking pathway for variant antigens destined for the surface of the Plasmodium falciparum-infected erythrocyte. Mol Biochem Parasit *133*, 1-14.

Hanspal, M., Goel, V.K., Oh, S.S., and Chishti, A.H. (2002). Erythrocyte calpain is dispensable for malaria parasite invasion and growth. Mol Biochem Parasit *122*, 227-229.

Harris, P.K., Yeoh, S., Dluzewski, A.R., O'Donnell, R.A., Withers-Martinez, C., Hackett, F., Bannister, L.H., Mitchell, G.H., and Blackman, M.J. (2005). Molecular identification of a malaria merozoite surface sheddase. PLoS pathogens *1*, 241-251.

Hasler, T., Handunnetti, S.M., Aguiar, J.C., van Schravendijk, M.R., Greenwood, B.M., Lallinger, G., Cegielski, P., and Howard, R.J. (1990). In vitro rosetting, cytoadherence,

and microagglutination properties of Plasmodium falciparum-infected erythrocytes from Gambian and Tanzanian patients. Blood *76*, 1845-1852.

Hay, S.I., Okiro, E.A., Gething, P.W., Patil, A.P., Tatem, A.J., Guerra, C.A., and Snow, R.W. (2010). Estimating the global clinical burden of Plasmodium falciparum malaria in 2007. PLoS medicine *7*, e1000290.

Healer, J., Crawford, S., Ralph, S., McFadden, G., and Cowman, A.F. (2002). Independent translocation of two micronemal proteins in developing Plasmodium falciparum merozoites. Infection and Immunity *70*, 5751-5758.

Healer, J., Murphy, V., Hodder, A.N., Masciantonio, R., Gemmill, A.W., Anders, R.F., Cowman, A.F., and Batchelor, A. (2004). Allelic polymorphisms in apical membrane antigen-1 are responsible for evasion of antibody-mediated inhibition in Plasmodium falciparum. Molecular microbiology *52*, 159-168.

Hearty, S., Conroy, P.J., Ayyar, B.V., Byrne, B., and O'Kennedy, R. (2010). Surface plasmon resonance for vaccine design and efficacy studies: recent applications and future trends. Expert review of vaccines *9*, 645-664.

Heddini, A., Keusch, G.T., and Davies, C.S. (2004). The multilateral initiative on malaria: past, present, and future. The American journal of tropical medicine and hygiene *71*, 279-282.

Heddini, A., Pettersson, F., Kai, O., Shafi, J., Obiero, J., Chen, Q., Barragan, A., Wahlgren, M., and Marsh, K. (2001). Fresh isolates from children with severe Plasmodium falciparum malaria bind to multiple receptors. Infection and Immunity *69*, 5849-5856.

Hiller, N.L., Bhattacharjee, S., van Ooij, C., Liolios, K., Harrison, T., Lopez-Estrano, C., and Haldar, K. (2004). A host-targeting signal in virulence proteins reveals a secretome in malarial infection. Science *306*, 1934-1937.

Hodder, A.N., Crewther, P.E., and Anders, R.F. (2001). Specificity of the protective antibody response to apical membrane antigen 1. Infection and Immunity *69*, 3286-3294.

Hodder, A.N., Drew, D.R., Epa, V.C., Delorenzi, M., Bourgon, R., Miller, S.K., Moritz, R.L., Frecklington, D.F., Simpson, R.J., Speed, T.P*., et al.* (2003). Enzymic, phylogenetic, and structural characterization of the unusual papain-like protease domain of Plasmodium falciparum SERA5. The Journal of biological chemistry *278*, 48169-48177.

Hoffmann, E.H., da Silveira, L.A., Tonhosolo, R., Pereira, F.J., Ribeiro, W.L., Tonon, A.P., Kawamoto, F., and Ferreira, M.U. (2001). Geographical patterns of allelic diversity in the Plasmodium falciparum malaria-vaccine candidate, merozoite surface protein-2. Ann Trop Med Parasit *95*, 117-132.

Holder, A.A. (1988). The precursor to major merozoite surface antigens: structure and role in immunity. Prog Allergy *41*, 72-97.

Holder, A.A., Blackman, M.J., Burghaus, P.A., Chappel, J.A., Ling, I.T., McCallum-Deighton, N., and Shai, S. (1992). A malaria merozoite surface protein (MSP1)-structure, processing and function. Mem I Oswaldo Cruz *87 Suppl 3*, 37-42.

Holder, A.A., and Freeman, R.R. (1981). Immunization against blood-stage rodent malaria using purified parasite antigens. Nature *294*, 361-364.

Holder, A.A., and Freeman, R.R. (1982). Biosynthesis and processing of a Plasmodium falciparum schizont antigen recognized by immune serum and a monoclonal antibody. The Journal of experimental medicine *156*, 1528-1538.

Hommel, M., David, P.H., and Oligino, L.D. (1983). Surface alterations of erythrocytes in Plasmodium falciparum malaria. Antigenic variation, antigenic diversity, and the role of the spleen. The Journal of experimental medicine *157*, 1137-1148.

Hoyer, L.L. (2001). The ALS gene family of Candida albicans. Trends in microbiology *9*, 176-180.

Hunt, N.H., and Grau, G.E. (2003). Cytokines: accelerators and brakes in the pathogenesis of cerebral malaria. Trends in immunology *24*, 491-499.

Idro, R., Karamagi, C., and Tumwine, J. (2004). Immediate outcome and prognostic factors for cerebral malaria among children admitted to Mulago Hospital, Uganda. Annals of tropical paediatrics *24*, 17-24.

Ingram, V.M. (1957). Gene mutations in human haemoglobin: the chemical difference between normal and sickle cell haemoglobin. Nature *180*, 326-328.

Iriemenam, N.C., Khirelsied, A.H., Nasr, A., ElGhazali, G., Giha, H.A., Elhassan, A.E.T.M., Agab-Aldour, A.A., Montgomery, S.M., Anders, R.F., Theisen, M*., et al.* (2009).

Antibody responses to a panel of Plasmodium falciparum malaria blood-stage antigens in relation to clinical disease outcome in Sudan. Vaccine *27*, 62-71.

Ismail, M.R., Ordi, J., Menendez, C., Ventura, P.J., Aponte, J.J., Kahigwa, E., Hirt, R., Cardesa, A., and Alonso, P.L. (2000). Placental pathology in malaria: a histological, immunohistochemical, and quantitative study. Hum Pathol *31*, 85-93.

Jakeman, G.N., Saul, A., Hogarth, W.L., and Collins, W.E. (1999). Anaemia of acute malaria infections in non-immune patients primarily results from destruction of uninfected erythrocytes. Parasitology *119 (Pt 2)*, 127-133.

Jallow, M., Teo, Y.Y., Small, K.S., Rockett, K.A., Deloukas, P., Clark, T.G., Kivinen, K., Bojang, K.A., Conway, D.J., Pinder, M.*, et al.* (2009). Genome-wide and fine-resolution association analysis of malaria in West Africa. Nature genetics *41*, 657-665.

Jensen, A.T., Magistrado, P., Sharp, S., Joergensen, L., Lavstsen, T., Chiucchiuini, A., Salanti, A., Vestergaard, L.S., Lusingu, J.P., Hermsen, R.*, et al.* (2004). Plasmodium falciparum associated with severe childhood malaria preferentially expresses PfEMP1 encoded by group A var genes. The Journal of experimental medicine *199*, 1179-1190.

Jiang, G., Daubenberger, C., Huber, W., Matile, H., Tanner, M., and Pluschke, G. (2000). Sequence diversity of the merozoite surface protein 1 of Plasmodium falciparum in clinical isolates from the Kilombero District, Tanzania. Acta tropica *74*, 51-61.

Joannin, N., Abhiman, S., Sonnhammer, E.L., and Wahlgren, M. (2008). Sub-grouping and sub-functionalization of the RIFIN multi-copy protein family. BMC Genomics *9*, 19.

John, C.C., Moormann, A.M., Pregibon, D.C., Sumba, P.O., McHugh, M.M., Narum, D.L., Lanar, D.E., Schluchter, M.D., and Kazura, J.W. (2005). Correlation of high levels of antibodies to multiple pre-erythrocytic Plasmodium falciparum antigens and protection from infection. The American journal of tropical medicine and hygiene *73*, 222-228.

John, C.C., O'Donnell, R.A., Sumba, P.O., Moormann, A.M., de Koning-Ward, T.F., King, C.L., Kazura, J.W., and Crabb, B.S. (2004). Evidence that invasion-inhibitory antibodies specific for the 19-kDa fragment of merozoite surface protein-1 (MSP-1 19) can play a protective role against blood-stage Plasmodium falciparum infection in individuals in a malaria endemic area of Africa. Journal of immunology *173*, 666-672.

Kappe, S.H., Buscaglia, C.A., and Nussenzweig, V. (2004). Plasmodium sporozoite molecular cell biology. Annu Rev Cell Dev Biol *20*, 29-59.

Kappe, S.H., Vaughan, A.M., Boddey, J.A., and Cowman, A.F. (2010). That was then but this is now: malaria research in the time of an eradication agenda. Science *328*, 862-866.

Kappes, B., Doerig, C.D., and Graeser, R. (1999). An overview of Plasmodium protein kinases. Parasitology today *15*, 449-454.

Kato, K., Mayer, D.C., Singh, S., Reid, M., and Miller, L.H. (2005). Domain III of Plasmodium falciparum apical membrane antigen 1 binds to the erythrocyte membrane protein Kx. P Natl Acad Sci USA *102*, 5552-5557.

Kaviratne, M., Khan, S.M., Jarra, W., and Preiser, P.R. (2002). Small variant STEVOR antigen is uniquely located within Maurer's clefts in Plasmodium falciparum-infected red blood cells. Eukaryotic cell *1*, 926-935.

Keh, C.E., Jha, A.R., Nzarubara, B., Lanar, D.E., Dutta, S., Theisen, M., Rosenthal, P.J., Dorsey, G., Nixon, D.F., and Greenhouse, B. (2012). Associations between antibodies to a panel of Plasmodium falciparum specific antigens and response to sub-optimal antimalarial therapy in Kampala, Uganda. PLoS One *7*, e52571.

Kern, P., Hemmer, C.J., Van Damme, J., Gruss, H.J., and Dietrich, M. (1989). Elevated tumor necrosis factor alpha and interleukin-6 serum levels as markers for complicated Plasmodium falciparum malaria. The American journal of medicine *87*, 139-143.

Kester, K.E., Cummings, J.F., Ofori-Anyinam, O., Ockenhouse, C.F., Krzych, U., Moris, P., Schwenk, R., Nielsen, R.A., Debebe, Z., Pinelis, E.*, et al.* (2009). Randomized, double-blind, phase 2a trial of falciparum malaria vaccines RTS,S/AS01B and RTS,S/AS02A in malaria-naive adults: safety, efficacy, and immunologic associates of protection. The Journal of infectious diseases *200*, 337-346.

Khattab, A., and Klinkert, M.Q. (2006). Maurer's clefts-restricted localization, orientation and export of a Plasmodium falciparum RIFIN. Traffic *7*, 1654-1665.

Khattab, A., and Meri, S. (2011). Exposure of the Plasmodium falciparum clonally variant STEVOR proteins on the merozoite surface. Malaria J *10*, 58.

Kilejian, A., Rashid, M.A., Aikawa, M., Aji, T., and Yang, Y.F. (1991). Selective association of a fragment of the knob protein with spectrin, actin and the red cell membrane. Mol Biochem Parasit *44*, 175-181.

Kinyanjui, S.M., Bull, P., Newbold, C.I., and Marsh, K. (2003). Kinetics of antibody responses to Plasmodium falciparum-infected erythrocyte variant surface antigens. The Journal of infectious diseases *187*, 667-674.

Kiwuwa, M.S., Ribacke, U., Moll, K., Byarugaba, J., Lundblom, K., Farnert, A., Fred, K., and Wahlgren, M. (2013). Genetic diversity of Plasmodium falciparum infections in mild and severe malaria of children from Kampala, Uganda. Parasitology research *112*, 1691-1700.

Knuepfer, E., Rug, M., Klonis, N., Tilley, L., and Cowman, A.F. (2005). Trafficking of the major virulence factor to the surface of transfected P. falciparum-infected erythrocytes. Blood *105*, 4078-4087.

Kochar, D.K., Shubhakaran, Kumawat, B.L., Kochar, S.K., Halwai, M., Makkar, R.K., Joshi, A., and Thanvi, I. (2002). Cerebral malaria in Indian adults: a prospective study of 441 patients from Bikaner, north-west India. The Journal of the Association of Physicians of India *50*, 234-241.

Kocken, C.H., Withers-Martinez, C., Dubbeld, M.A., van der Wel, A., Hackett, F., Valderrama, A., Blackman, M.J., and Thomas, A.W. (2002). High-level expression of the malaria blood-stage vaccine candidate Plasmodium falciparum apical membrane antigen 1 and induction of antibodies that inhibit erythrocyte invasion. Infection and Immunity *70*, 4471-4476.

Kraemer, S.M., Kyes, S.A., Aggarwal, G., Springer, A.L., Nelson, S.O., Christodoulou, Z., Smith, L.M., Wang, W., Levin, E., Newbold, C.I.*, et al.* (2007). Patterns of gene recombination shape var gene repertoires in Plasmodium falciparum: comparisons of geographically diverse isolates. BMC Genomics *8*, 45.

Kriek, N., Tilley, L., Horrocks, P., Pinches, R., Elford, B.C., Ferguson, D.J., Lingelbach, K., and Newbold, C.I. (2003). Characterization of the pathway for transport of the cytoadherence-mediating protein, PfEMP1, to the host cell surface in malaria parasite-infected erythrocytes. Molecular microbiology *50*, 1215-1227.

Krych-Goldberg, M., Moulds, J.M., and Atkinson, J.P. (2002). Human complement receptor type 1 (CR1) binds to a major malarial adhesin. Trends in molecular medicine *8*, 531-537.

Kusi, K.A., Faber, B.W., Thomas, A.W., and Remarque, E.J. (2009). Humoral immune response to mixed PfAMA1 alleles; multivalent PfAMA1 vaccines induce broad specificity. PLoS One *4*, e8110.

Kwiatkowski, D. (1990). Tumour necrosis factor, fever and fatality in falciparum malaria. Immunology letters *25*, 213-216.

Kyes, S.A., Rowe, J.A., Kriek, N., and Newbold, C.I. (1999). Rifins: a second family of clonally variant proteins expressed on the surface of red cells infected with Plasmodium falciparum. P Natl Acad Sci USA *96*, 9333-9338.

Lamikanra, A.A., Brown, D., Potocnik, A., Casals-Pascual, C., Langhorne, J., and Roberts, D.J. (2007). Malarial anemia: of mice and men. Blood *110*, 18-28.

Lamikanra, A.A., Theron, M., Kooij, T.W., and Roberts, D.J. (2009). Hemozoin (malarial pigment) directly promotes apoptosis of erythroid precursors. PLoS One *4*, e8446.

Langhorne, J., Ndungu, F.M., Sponaas, A.M., and Marsh, K. (2008). Immunity to malaria: more questions than answers. Nature immunology *9*, 725-732.

Langreth, S.G., Jensen, J.B., Reese, R.T., and Trager, W. (1978). Fine structure of human malaria in vitro. The Journal of protozoology *25*, 443-452.

Lanzillotti, R., and Coetzer, T.L. (2004). Myosin-like sequences in the malaria parasite Plasmodium falciparum bind human erythrocyte membrane protein 4.1. Haematologica *89*, 1168-1171.

Lauer, S.A., Rathod, P.K., Ghori, N., and Haldar, K. (1997). A membrane network for nutrient import in red cells infected with the malaria parasite. Science *276*, 1122-1125.

Lauterbach, S.B., Lanzillotti, R., and Coetzer, T.L. (2003). Construction and use of Plasmodium falciparum phage display libraries to identify host parasite interactions. Malaria J *2*, 47.

Lavazec, C., Sanyal, S., and Templeton, T.J. (2006). Hypervariability within the Rifin, Stevor and Pfmc-2TM superfamilies in Plasmodium falciparum. Nucleic acids research *34*, 6696-6707.

Lavstsen, T., Salanti, A., Jensen, A.T., Arnot, D.E., and Theander, T.G. (2003). Sub-grouping of Plasmodium falciparum 3D7 var genes based on sequence analysis of coding and non-coding regions. Malaria J *2*, 27.

Lawson, C., and Wolf, S. (2009). ICAM-1 signaling in endothelial cells. Pharmacol Rep *61*, 22-32.

Lell, B., May, J., Schmidt-Ott, R.J., Lehman, L.G., Luckner, D., Greve, B., Matousek, P., Schmid, D., Herbich, K., Mockenhaupt, F.P.*, et al.* (1999). The role of red blood cell polymorphisms in resistance and susceptibility to malaria. Clinical infectious diseases : an official publication of the Infectious Diseases Society of America *28*, 794-799.

Leoratti, F.M., Durlacher, R.R., Lacerda, M.V., Alecrim, M.G., Ferreira, A.W., Sanchez, M.C., and Moraes, S.L. (2008). Pattern of humoral immune response to Plasmodium falciparum blood stages in individuals presenting different clinical expressions of malaria. Malaria J *7*, 186.

Lew, V.L., and Tiffert, T. (2007). Is invasion efficiency in malaria controlled by pre-invasion events? Trends in parasitology *23*, 481-484.

Li, X., Chen, H., Oo, T.H., Daly, T.M., Bergman, L.W., Liu, S.C., Chishti, A.H., and Oh, S.S. (2004). A co-ligand complex anchors Plasmodium falciparum merozoites to the erythrocyte invasion receptor band 3. The Journal of biological chemistry *279*, 5765-5771.

Lopaticki, S., Maier, A.G., Thompson, J., Wilson, D.W., Tham, W.H., Triglia, T., Gout, A., Speed, T.P., Beeson, J.G., Healer, J.*, et al.* (2011). Reticulocyte and erythrocyte binding-like proteins function cooperatively in invasion of human erythrocytes by malaria parasites. Infection and Immunity *79*, 1107-1117.

Loscertales, M.P., Owens, S., O'Donnell, J., Bunn, J., Bosch-Capblanch, X., and Brabin, B.J. (2007). ABO blood group phenotypes and Plasmodium falciparum malaria: unlocking a pivotal mechanism. Advances in parasitology *65*, 1-50.

Maier, A.G., Baum, J., Smith, B., Conway, D.J., and Cowman, A.F. (2009a). Polymorphisms in erythrocyte binding antigens 140 and 181 affect function and binding but not receptor specificity in Plasmodium falciparum. Infection and Immunity *77*, 1689-1699.

Maier, A.G., Cooke, B.M., Cowman, A.F., and Tilley, L. (2009b). Malaria parasite proteins that remodel the host erythrocyte. Nature reviews Microbiology *7*, 341-354.

Maier, A.G., Duraisingh, M.T., Reeder, J.C., Patel, S.S., Kazura, J.W., Zimmerman, P.A., and Cowman, A.F. (2003). Plasmodium falciparum erythrocyte invasion through glycophorin C and selection for Gerbich negativity in human populations. Nature Medicine *9*, 87-92.

Maier, A.G., Rug, M., O'Neill, M.T., Beeson, J.G., Marti, M., Reeder, J., and Cowman, A.F. (2007). Skeleton-binding protein 1 functions at the parasitophorous vacuole membrane to traffic PfEMP1 to the Plasmodium falciparum-infected erythrocyte surface. Blood *109*, 1289-1297.

Malaney, P., Spielman, A., and Sachs, J. (2004). The malaria gap. The American journal of tropical medicine and hygiene *71*, 141-146.

Malkin, E., Hu, J., Li, Z., Chen, Z., Bi, X., Reed, Z., Dubovsky, F., Liu, J., Wang, Q., Pan, X.*, et al.* (2008). A phase 1 trial of PfCP2.9: an AMA1/MSP1 chimeric recombinant protein vaccine for Plasmodium falciparum malaria. Vaccine *26*, 6864-6873.

Marsh, K., Forster, D., Waruiru, C., Mwangi, I., Winstanley, M., Marsh, V., Newton, C., Winstanley, P., Warn, P., Peshu, N.*, et al.* (1995). Indicators of life-threatening malaria in African children. The New England journal of medicine *332*, 1399-1404.

Marsh, K., and Kinyanjui, S. (2006). Immune effector mechanisms in malaria. Parasite immunology *28*, 51-60.

Marshall, V.M., Peterson, M.G., Lew, A.M., and Kemp, D.J. (1989). Structure of the apical membrane antigen I (AMA-1) of Plasmodium chabaudi. Mol Biochem Parasit *37*, 281-283.

Marti, M., Good, R.T., Rug, M., Knuepfer, E., and Cowman, A.F. (2004). Targeting malaria virulence and remodeling proteins to the host erythrocyte. Science *306*, 1930-1933.

Martiney, J.A., Sherry, B., Metz, C.N., Espinoza, M., Ferrer, A.S., Calandra, T., Broxmeyer, H.E., and Bucala, R. (2000). Macrophage migration inhibitory factor release by

macrophages after ingestion of Plasmodium chabaudi-infected erythrocytes: possible role in the pathogenesis of malarial anemia. Infection and Immunity *68*, 2259-2267.

Maubert, B., Fievet, N., Tami, G., Boudin, C., and Deloron, P. (2000). Cytoadherence of Plasmodium falciparum-infected erythrocytes in the human placenta. Parasite immunology *22*, 191-199.

May, J., Evans, J.A., Timmann, C., Ehmen, C., Busch, W., Thye, T., Agbenyega, T., and Horstmann, R.D. (2007). Hemoglobin variants and disease manifestations in severe falciparum malaria. JAMA : the journal of the American Medical Association *297*, 2220-2226.

Mayer, D.C., Jiang, L., Achur, R.N., Kakizaki, I., Gowda, D.C., and Miller, L.H. (2006). The glycophorin C N-linked glycan is a critical component of the ligand for the Plasmodium falciparum erythrocyte receptor BAEBL. P Natl Acad Sci USA *103*, 2358-2362.

Mayer, D.C., Mu, J.B., Feng, X., Su, X.Z., and Miller, L.H. (2002). Polymorphism in a Plasmodium falciparum erythrocyte-binding ligand changes its receptor specificity. The Journal of experimental medicine *196*, 1523-1528.

Mayer, D.C., Mu, J.B., Kaneko, O., Duan, J., Su, X.Z., and Miller, L.H. (2004). Polymorphism in the Plasmodium falciparum erythrocyte-binding ligand JESEBL/EBA-181 alters its receptor specificity. P Natl Acad Sci USA *101*, 2518-2523.

McCallum, F.J., Persson, K.E., Mugyenyi, C.K., Fowkes, F.J., Simpson, J.A., Richards, J.S., Williams, T.N., Marsh, K., and Beeson, J.G. (2008). Acquisition of growth-inhibitory antibodies against blood-stage Plasmodium falciparum. PLoS One *3*, e3571.

McCarra, M.B., Ayodo, G., Sumba, P.O., Kazura, J.W., Moormann, A.M., Narum, D.L., and John, C.C. (2011). Antibodies to Plasmodium falciparum erythrocyte-binding antigen-175 are associated with protection from clinical malaria. The Pediatric infectious disease journal *30*, 1037-1042.

McGregor, I.A., Williams, K., and Goodwin, L.G. (1963). Pyrimethamine and Sulphadiazine in Treatment of Malaria. Brit Med J *2*, 728-729.

McIntosh, R.S., Shi, J., Jennings, R.M., Chappel, J.C., de Koning-Ward, T.F., Smith, T., Green, J., van Egmond, M., Leusen, J.H., Lazarou, M.*, et al.* (2007). The importance of human FcgammaRI in mediating protection to malaria. PLoS pathogens *3*, e72.

Metzger, W.G., Okenu, D.M., Cavanagh, D.R., Robinson, J.V., Bojang, K.A., Weiss, H.A., McBride, J.S., Greenwood, B.M., and Conway, D.J. (2003). Serum IgG3 to the Plasmodium falciparum merozoite surface protein 2 is strongly associated with a reduced prospective risk of malaria. Parasite immunology *25*, 307-312.

Miller, L.H., Ackerman, H.C., Su, X.Z., and Wellems, T.E. (2013). Malaria biology and disease pathogenesis: insights for new treatments. Nature Medicine *19*, 156-167.

Miller, L.H., Baruch, D.I., Marsh, K., and Doumbo, O.K. (2002a). The pathogenic basis of malaria. Nature *415*, 673-679.

Miller, L.H., McAuliffe, F.M., and Mason, S.J. (1977). Erythrocyte receptors for malaria merozoites. The American journal of tropical medicine and hygiene *26*, 204-208.

Miller, L.H., Roberts, T., Shahabuddin, M., and McCutchan, T.F. (1993). Analysis of sequence diversity in the Plasmodium falciparum merozoite surface protein-1 (MSP-1). Mol Biochem Parasit *59*, 1-14.

Miller, S.K., Good, R.T., Drew, D.R., Delorenzi, M., Sanders, P.R., Hodder, A.N., Speed, T.P., Cowman, A.F., de Koning-Ward, T.F., and Crabb, B.S. (2002b). A subset of Plasmodium falciparum SERA genes are expressed and appear to play an important role in the erythrocytic cycle. The Journal of biological chemistry *277*, 47524-47532.

Mital, J., Meissner, M., Soldati, D., and Ward, G.E. (2005). Conditional expression of Toxoplasma gondii apical membrane antigen-1 (TgAMA1) demonstrates that TgAMA1 plays a critical role in host cell invasion. Molecular biology of the cell *16*, 4341-4349.

Mitchell, G.H., Hadley, T.J., McGinniss, M.H., Klotz, F.W., and Miller, L.H. (1986). Invasion of erythrocytes by Plasmodium falciparum malaria parasites: evidence for receptor heterogeneity and two receptors. Blood *67*, 1519-1521.

Mitchell, G.H., Thomas, A.W., Margos, G., Dluzewski, A.R., and Bannister, L.H. (2004). Apical membrane antigen 1, a major malaria vaccine candidate, mediates the close attachment of invasive merozoites to host red blood cells. Infection and Immunity *72*, 154-158.

Moll K, L.I., Perlmann H, Scherf A & Wahlgren M (Eds.) (2008). Methods in Malaria Research, 5th edn.

Molyneux, M.E., Taylor, T.E., Wirima, J.J., and Borgstein, A. (1989). Clinical features and prognostic indicators in paediatric cerebral malaria: a study of 131 comatose Malawian children. The Quarterly journal of medicine *71*, 441-459.

Molyneux, M.E., Taylor, T.E., Wirima, J.J., and Grau, G.E. (1991). Tumour necrosis factor, interleukin-6, and malaria. Lancet *337*, 1098.

Mota, M.M., Hafalla, J.C., and Rodriguez, A. (2002). Migration through host cells activates Plasmodium sporozoites for infection. Nature Medicine *8*, 1318-1322.

Mota, M.M., Pradel, G., Vanderberg, J.P., Hafalla, J.C., Frevert, U., Nussenzweig, R.S., Nussenzweig, V., and Rodriguez, A. (2001). Migration of Plasmodium sporozoites through cells before infection. Science *291*, 141-144.

Mphande, F.A., Ribacke, U., Kaneko, O., Kironde, F., Winter, G., and Wahlgren, M. (2008). SURFIN4.1, a schizont-merozoite associated protein in the SURFIN family of Plasmodium falciparum. Malaria J *7*, 116.

Mugyenyi, C.K., Elliott, S.R., McCallum, F.J., Anders, R.F., Marsh, K., and Beeson, J.G. (2013). Antibodies to polymorphic invasion-inhibitory and non-Inhibitory epitopes of Plasmodium falciparum apical membrane antigen 1 in human malaria. PLoS One *8*, e68304.

Murphy, S.C., and Breman, J.G. (2001). Gaps in the childhood malaria burden in Africa: cerebral malaria, neurological sequelae, anemia, respiratory distress, hypoglycemia, and complications of pregnancy. The American journal of tropical medicine and hygiene *64*, 57-67.

Murray, P.R., Rosenthal K.S., Pfaller M.A. (2009). Medical Microbiology, Sixth edition edn (Philadelphia, PA, Mosby Elsevier).

Nair, M., Hinds, M.G., Coley, A.M., Hodder, A.N., Foley, M., Anders, R.F., and Norton, R.S. (2002). Structure of domain III of the blood-stage malaria vaccine candidate, Plasmodium falciparum apical membrane antigen 1 (AMA1). Journal of molecular biology *322*, 741-753.

Narum, D.L., Ogun, S.A., Batchelor, A.H., and Holder, A.A. (2006). Passive immunization with a multicomponent vaccine against conserved domains of apical membrane antigen 1 and 235-kilodalton rhoptry proteins protects mice against Plasmodium yoelii blood-stage challenge infection. Infection and Immunity *74*, 5529-5536.

Narum, D.L., and Thomas, A.W. (1994). Differential localization of full-length and processed forms of PF83/AMA-1 an apical membrane antigen of Plasmodium falciparum merozoites. Mol Biochem Parasit *67*, 59-68.

Nasr, A., Iriemenam, N.C., Giha, H.A., Balogun, H.A., Anders, R.F., Troye-Blomberg, M., ElGhazali, G., and Berzins, K. (2009). FcgammaRIIa (CD32) polymorphism and anti-malarial IgG subclass pattern among Fulani and sympatric ethnic groups living in eastern Sudan. Malaria J *8*, 43.

Nery, S., Deans, A.M., Mosobo, M., Marsh, K., Rowe, J.A., and Conway, D.J. (2006). Expression of Plasmodium falciparum genes involved in erythrocyte invasion varies among isolates cultured directly from patients. Mol Biochem Parasit *149*, 208-215.

Newbold, C., Warn, P., Black, G., Berendt, A., Craig, A., Snow, B., Msobo, M., Peshu, N., and Marsh, K. (1997). Receptor-specific adhesion and clinical disease in Plasmodium falciparum. The American journal of tropical medicine and hygiene *57*, 389-398.

Newton, C.R., Hien, T.T., and White, N. (2000). Cerebral malaria. Journal of neurology, neurosurgery, and psychiatry *69*, 433-441.

Ngoungou, E.B., and Preux, P.M. (2008). Cerebral malaria and epilepsy. Epilepsia *49 Suppl 6*, 19-24.

Niang, M., Yan Yam, X., and Preiser, P.R. (2009). The Plasmodium falciparum STEVOR multigene family mediates antigenic variation of the infected erythrocyte. PLoS pathogens *5*, e1000307.

Nimmerjahn, F., and Ravetch, J.V. (2008). Anti-inflammatory actions of intravenous immunoglobulin. Annual review of immunology *26*, 513-533.

Noland, G.S., Hendel-Paterson, B., Min, X.M., Moormann, A.M., Vulule, J.M., Narum, D.L., Lanar, D.E., Kazura, J.W., and John, C.C. (2008). Low prevalence of antibodies to

preerythrocytic but not blood-stage Plasmodium falciparum antigens in an area of unstable malaria transmission compared to prevalence in an area of stable malaria transmission. Infection and Immunity *76*, 5721-5728.

Normark, J., Nilsson, D., Ribacke, U., Winter, G., Moll, K., Wheelock, C.E., Bayarugaba, J., Kironde, F., Egwang, T.G., Chen, Q.*, et al.* (2007). PfEMP1-DBL1alpha amino acid motifs in severe disease states of Plasmodium falciparum malaria. P Natl Acad Sci USA *104*, 15835-15840.

Noutcha, M.A., and Anumdu, C.I. (2009). Entomological indices of Anopheles gambiae sensu lato at a rural community in south-west Nigeria. J Vector Dis *46*, 43-51.

Ntoumi, F., Contamin, H., Rogier, C., Bonnefoy, S., Trape, J.F., and Mercereau-Puijalon, O. (1995). Age-dependent carriage of multiple Plasmodium falciparum merozoite surface antigen-2 alleles in asymptomatic malaria infections. The American journal of tropical medicine and hygiene *52*, 81-88.

Ochola, L.B., Siddondo, B.R., Ocholla, H., Nkya, S., Kimani, E.N., Williams, T.N., Makale, J.O., Liljander, A., Urban, B.C., Bull, P.C.*, et al.* (2011). Specific receptor usage in Plasmodium falciparum cytoadherence is associated with disease outcome. PLoS One *6*, e14741.

Ockenhouse, C.F., Klotz, F.W., Tandon, N.N., and Jamieson, G.A. (1991). Sequestrin, a CD36 recognition protein on Plasmodium falciparum malaria-infected erythrocytes identified by anti-idiotype antibodies. P Natl Acad Sci USA *88*, 3175-3179.

Ogutu, B.R., Apollo, O.J., McKinney, D., Okoth, W., Siangla, J., Dubovsky, F., Tucker, K., Waitumbi, J.N., Diggs, C., Wittes, J.*, et al.* (2009). Blood stage malaria vaccine eliciting high antigen-specific antibody concentrations confers no protection to young children in Western Kenya. PLoS One *4*, e4708.

Oh, S.S., Voigt, S., Fisher, D., Yi, S.J., LeRoy, P.J., Derick, L.H., Liu, S., and Chishti, A.H. (2000). Plasmodium falciparum erythrocyte membrane protein 1 is anchored to the actin-spectrin junction and knob-associated histidine-rich protein in the erythrocyte skeleton. Mol Biochem Parasit *108*, 237-247.

Okello, P.E., Van Bortel, W., Byaruhanga, A.M., Correwyn, A., Roelants, P., Talisuna, A., D'Alessandro, U., and Coosemans, M. (2006). Variation in malaria transmission intensity in seven sites throughout Uganda. The American journal of tropical medicine and hygiene *75*, 219-225.

Okenu, D.M., Riley, E.M., Bickle, Q.D., Agomo, P.U., Barbosa, A., Daugherty, J.R., Lanar, D.E., and Conway, D.J. (2000). Analysis of human antibodies to erythrocyte binding antigen 175 of Plasmodium falciparum. Infection and Immunity *68*, 5559-5566.

Olotu, A., Lusingu, J., Leach, A., Lievens, M., Vekemans, J., Msham, S., Lang, T., Gould, J., Dubois, M.C., Jongert, E.*, et al.* (2011). Efficacy of RTS,S/AS01E malaria vaccine and exploratory analysis on anti-circumsporozoite antibody titres and protection in children aged 5-17 months in Kenya and Tanzania: a randomised controlled trial. The Lancet infectious diseases *11*, 102-109.

Orlandi, P.A., Sim, B.K., Chulay, J.D., and Haynes, J.D. (1990). Characterization of the 175-kilodalton erythrocyte binding antigen of Plasmodium falciparum. Mol Biochem Parasit *40*, 285-294.

Osier, F.H., Fegan, G., Polley, S.D., Murungi, L., Verra, F., Tetteh, K.K., Lowe, B., Mwangi, T., Bull, P.C., Thomas, A.W.*, et al.* (2008). Breadth and magnitude of antibody responses to multiple Plasmodium falciparum merozoite antigens are associated with protection from clinical malaria. Infection and Immunity *76*, 2240-2248.

Pachebat, J.A., Ling, I.T., Grainger, M., Trucco, C., Howell, S., Fernandez-Reyes, D., Gunaratne, R., and Holder, A.A. (2001). The 22 kDa component of the protein complex on the surface of Plasmodium falciparum merozoites is derived from a larger precursor, merozoite surface protein 7. Mol Biochem Parasit *117*, 83-89.

Pasvol, G. (2003). How many pathways for invasion of the red blood cell by the malaria parasite? Trends in parasitology *19*, 430-432.

Pasvol, G., Weatherall, D.J., and Wilson, R.J. (1978). Cellular mechanism for the protective effect of haemoglobin S against P. falciparum malaria. Nature *274*, 701-703.

Pathirana, S.L., Alles, H.K., Bandara, S., Phone-Kyaw, M., Perera, M.K., Wickremasinghe, A.R., Mendis, K.N., and Handunnetti, S.M. (2005). ABO-blood-group types and

protection against severe, Plasmodium falciparum malaria. Ann Trop Med Parasit *99*, 119-124.

Perraut, R., Marrama, L., Diouf, B., Sokhna, C., Tall, A., Nabeth, P., Trape, J.F., Longacre, S., and Mercereau-Puijalon, O. (2005). Antibodies to the conserved C-terminal domain of the Plasmodium falciparum merozoite surface protein 1 and to the merozoite extract and their relationship with in vitro inhibitory antibodies and protection against clinical malaria in a Senegalese village. The Journal of infectious diseases *191*, 264-271.

Persson, K.E.M, Fowkes, F.J., McCallum, F.J., Gicheru, N., Reiling, L., Richards, J.S., Wilson, D.W., Lopaticki, S., Cowman, A.F., Marsh, K.*, et al.* (2013). Erythrocyte-binding antigens of Plasmodium falciparum are targets of human inhibitory antibodies and function to evade naturally acquired immunity. Journal of immunology *191*, 785-794.

Persson, K.E.M, Lee, C.T., Marsh, K., and Beeson, J.G. (2006). Development and optimization of high-throughput methods to measure Plasmodium falciparum-specific growth inhibitory antibodies. Journal of clinical microbiology *44*, 1665-1673.

Persson, K.E.M, McCallum, F.J., Reiling, L., Lister, N.A., Stubbs, J., Cowman, A.F., Marsh, K., and Beeson, J.G. (2008). Variation in use of erythrocyte invasion pathways by Plasmodium falciparum mediates evasion of human inhibitory antibodies. The Journal of clinical investigation *118*, 342-351.

Peterson, D.S., and Wellems, T.E. (2000). EBL-1, a putative erythrocyte binding protein of Plasmodium falciparum, maps within a favored linkage group in two genetic crosses. Mol Biochem Parasit *105*, 105-113.

Peterson MG, M.V.M., Smythe JA, Crewther PE, Lew A, Silva A, Anders R F, Kemp D J (1989). Integral membrane protein located in the apical complex of Plasmodium falciparum. Molecular and Cellular Biology *9*, 3151-3154.

Petter, M., Bonow, I., and Klinkert, M.Q. (2008). Diverse expression patterns of subgroups of the rif multigene family during Plasmodium falciparum gametocytogenesis. PLoS One *3*, e3779.

Petter, M., Haeggstrom, M., Khattab, A., Fernandez, V., Klinkert, M.Q., and Wahlgren, M. (2007). Variant proteins of the Plasmodium falciparum RIFIN family show distinct subcellular localization and developmental expression patterns. Mol Biochem Parasit *156*, 51-61.

Pimenta, P.F., Touray, M., and Miller, L. (1994). The journey of malaria sporozoites in the mosquito salivary gland. The Journal of eukaryotic microbiology *41*, 608-624.

Pizarro, J.C., Vulliez-Le Normand, B., Chesne-Seck, M.L., Collins, C.R., Withers-Martinez, C., Hackett, F., Blackman, M.J., Faber, B.W., Remarque, E.J., Kocken, C.H.*, et al.* (2005). Crystal structure of the malaria vaccine candidate apical membrane antigen 1. Science *308*, 408-411.

Polley, S.D., and Conway, D.J. (2001). Strong diversifying selection on domains of the Plasmodium falciparum apical membrane antigen 1 gene. Genetics *158*, 1505-1512.

Polley, S.D., Mwangi, T., Kocken, C.H., Thomas, A.W., Dutta, S., Lanar, D.E., Remarque, E., Ross, A., Williams, T.N., Mwambingu, G.*, et al.* (2004). Human antibodies to recombinant protein constructs of Plasmodium falciparum Apical Membrane Antigen 1 (AMA1) and their associations with protection from malaria. Vaccine *23*, 718-728.

Pombo, D.J., Lawrence, G., Hirunpetcharat, C., Rzepczyk, C., Bryden, M., Cloonan, N., Anderson, K., Mahakunkijcharoen, Y., Martin, L.B., Wilson, D.*, et al.* (2002). Immunity to malaria after administration of ultra-low doses of red cells infected with Plasmodium falciparum. Lancet *360*, 610-617.

Pongponratn, E., Riganti, M., Punpoowong, B., and Aikawa, M. (1991). Microvascular sequestration of parasitized erythrocytes in human falciparum malaria: a pathological study. The American journal of tropical medicine and hygiene *44*, 168-175.

Pradel, G., and Frevert, U. (2001). Malaria sporozoites actively enter and pass through rat Kupffer cells prior to hepatocyte invasion. Hepatology *33*, 1154-1165.

Preiser, P.R., Khan, S., Costa, F.T., Jarra, W., Belnoue, E., Ogun, S., Holder, A.A., Voza, T., Landau, I., Snounou, G.*, et al.* (2002). Stage-specific transcription of distinct repertoires of a multigene family during Plasmodium life cycle. Science *295*, 342-345.

Prescott, N., Stowers, A.W., Cheng, Q., Bobogare, A., Rzepczyk, C.M., and Saul, A. (1994). Plasmodium falciparum genetic diversity can be characterised using the polymorphic

merozoite surface antigen 2 (MSA-2) gene as a single locus marker. Mol Biochem Parasit *63*, 203-212.
Price, R.N., Douglas, N.M., and Anstey, N.M. (2009). New developments in Plasmodium vivax malaria: severe disease and the rise of chloroquine resistance. Current opinion in infectious diseases *22*, 430-435.
Price, R.N., Tjitra, E., Guerra, C.A., Yeung, S., White, N.J., and Anstey, N.M. (2007). Vivax malaria: neglected and not benign. The American journal of tropical medicine and hygiene *77*, 79-87.
Rasti, N., Namusoke, F., Chene, A., Chen, Q., Staalsoe, T., Klinkert, M.Q., Mirembe, F., Kironde, F., and Wahlgren, M. (2006). Nonimmune immunoglobulin binding and multiple adhesion characterize Plasmodium falciparum-infected erythrocytes of placental origin. P Natl Acad Sci USA *103*, 13795-13800.
Rayner, J.C., Galinski, M.R., Ingravallo, P., and Barnwell, J.W. (2000). Two Plasmodium falciparum genes express merozoite proteins that are related to Plasmodium vivax and Plasmodium yoelii adhesive proteins involved in host cell selection and invasion. P Natl Acad Sci USA *97*, 9648-9653.
Reddy, S.B., Anders, R.F., Beeson, J.G., Farnert, A., Kironde, F., Berenzon, S.K., Wahlgren, M., Linse, S., and Persson, K.E.M (2012). High affinity antibodies to Plasmodium falciparum merozoite antigens are associated with protection from malaria. PLoS One *7*, e32242.
Reed, M.B., Caruana, S.R., Batchelor, A.H., Thompson, J.K., Crabb, B.S., and Cowman, A.F. (2000). Targeted disruption of an erythrocyte binding antigen in Plasmodium falciparum is associated with a switch toward a sialic acid-independent pathway of invasion. P Natl Acad Sci USA *97*, 7509-7514.
Reiling, L., Richards, J.S., Fowkes, F.J., Barry, A.E., Triglia, T., Chokejindachai, W., Michon, P., Tavul, L., Siba, P.M., Cowman, A.F.*, et al.* (2010). Evidence that the erythrocyte invasion ligand PfRh2 is a target of protective immunity against Plasmodium falciparum malaria. Journal of immunology *185*, 6157-6167.
Reyes-Sandoval, A., Berthoud, T., Alder, N., Siani, L., Gilbert, S.C., Nicosia, A., Colloca, S., Cortese, R., and Hill, A.V. (2010). Prime-boost immunization with adenoviral and modified vaccinia virus Ankara vectors enhances the durability and polyfunctionality of protective malaria CD8+ T-cell responses. Infection and Immunity *78*, 145-153.
Ribacke, U., Mok, B.W., Wirta, V., Normark, J., Lundeberg, J., Kironde, F., Egwang, T.G., Nilsson, P., and Wahlgren, M. (2007). Genome wide gene amplifications and deletions in Plasmodium falciparum. Mol Biochem Parasit *155*, 33-44.
Richards, J.S., Stanisic, D.I., Fowkes, F.J., Tavul, L., Dabod, E., Thompson, J.K., Kumar, S., Chitnis, C.E., Narum, D.L., Michon, P.*, et al.* (2010). Association between naturally acquired antibodies to erythrocyte-binding antigens of Plasmodium falciparum and protection from malaria and high-density parasitemia. Clinical infectious diseases : an official publication of the Infectious Diseases Society of America *51*, e50-60.
Riley, E.M., Allen, S.J., Wheeler, J.G., Blackman, M.J., Bennett, S., Takacs, B., Schonfeld, H.J., Holder, A.A., and Greenwood, B.M. (1992). Naturally acquired cellular and humoral immune responses to the major merozoite surface antigen (PfMSP1) of Plasmodium falciparum are associated with reduced malaria morbidity. Parasite immunology *14*, 321-337.
Rogerson, S.J., Hviid, L., Duffy, P.E., Leke, R.F., and Taylor, D.W. (2007). Malaria in pregnancy: pathogenesis and immunity. The Lancet infectious diseases *7*, 105-117.
Rogerson, S.J., Pollina, E., Getachew, A., Tadesse, E., Lema, V.M., and Molyneux, M.E. (2003). Placental monocyte infiltrates in response to Plasmodium falciparum malaria infection and their association with adverse pregnancy outcomes. The American journal of tropical medicine and hygiene *68*, 115-119.
Rogerson, S.J., Tembenu, R., Dobano, C., Plitt, S., Taylor, T.E., and Molyneux, M.E. (1999). Cytoadherence characteristics of Plasmodium falciparum-infected erythrocytes from Malawian children with severe and uncomplicated malaria. The American journal of tropical medicine and hygiene *61*, 467-472.
Romi, R., Razaiarimanga, M.C., Raharimanga, R., Rakotondraibe, E.M., Ranaivo, L.H., Pietra, V., Raveloson, A., and Majori, G. (2002). Impact of the malaria control campaign (1993-

1998) in the highlands of Madagascar: parasitological and entomological data. The American journal of tropical medicine and hygiene *66*, 2-6.
Roussilhon, C., Oeuvray, C., Muller-Graf, C., Tall, A., Rogier, C., Trape, J.F., Theisen, M., Balde, A., Perignon, J.L., and Druilhe, P. (2007). Long-term clinical protection from falciparum malaria is strongly associated with IgG3 antibodies to merozoite surface protein 3. PLoS medicine *4*, e320.
Rowe, A., Berendt, A.R., Marsh, K., and Newbold, C.I. (1994). Plasmodium falciparum: a family of sulphated glycoconjugates disrupts erythrocyte rosettes. Experimental parasitology *79*, 506-516.
Rowe, A., Obeiro, J., Newbold, C.I., and Marsh, K. (1995). Plasmodium falciparum rosetting is associated with malaria severity in Kenya. Infection and Immunity *63*, 2323-2326.
Rowe, J.A., Handel, I.G., Thera, M.A., Deans, A.M., Lyke, K.E., Kone, A., Diallo, D.A., Raza, A., Kai, O., Marsh, K., *et al.* (2007). Blood group O protects against severe Plasmodium falciparum malaria through the mechanism of reduced rosetting. P Natl Acad Sci USA *104*, 17471-17476.
Rowe, J.A., Kyes, S.A., Rogerson, S.J., Babiker, H.A., and Raza, A. (2002a). Identification of a conserved Plasmodium falciparum var gene implicated in malaria in pregnancy. The Journal of infectious diseases *185*, 1207-1211.
Rowe, J.A., Moulds, J.M., Newbold, C.I., and Miller, L.H. (1997). P. falciparum rosetting mediated by a parasite-variant erythrocyte membrane protein and complement-receptor 1. Nature *388*, 292-295.
Rowe, J.A., Rogerson, S.J., Raza, A., Moulds, J.M., Kazatchkine, M.D., Marsh, K., Newbold, C.I., Atkinson, J.P., and Miller, L.H. (2000). Mapping of the region of complement receptor (CR) 1 required for Plasmodium falciparum rosetting and demonstration of the importance of CR1 in rosetting in field isolates. Journal of immunology *165*, 6341-6346.
Rowe, J.A., Shafi, J., Kai, O.K., Marsh, K., and Raza, A. (2002b). Nonimmune IgM, but not IgG binds to the surface of Plasmodium falciparum-infected erythrocytes and correlates with rosetting and severe malaria. The American journal of tropical medicine and hygiene *66*, 692-699.
Roy, S.W., Ferreira, M.U., and Hartl, D.L. (2008). Evolution of allelic dimorphism in malarial surface antigens. Heredity *100*, 103-110.
Sachs, J., and Malaney, P. (2002). The economic and social burden of malaria. Nature *415*, 680-685.
Sachs, J.D. (2002). A new global effort to control malaria. Science *298*, 122-124.
Salanti, A., Dahlback, M., Turner, L., Nielsen, M.A., Barfod, L., Magistrado, P., Jensen, A.T., Lavstsen, T., Ofori, M.F., Marsh, K., *et al.* (2004). Evidence for the involvement of VAR2CSA in pregnancy-associated malaria. The Journal of experimental medicine *200*, 1197-1203.
Salanti, A., Staalsoe, T., Lavstsen, T., Jensen, A.T., Sowa, M.P., Arnot, D.E., Hviid, L., and Theander, T.G. (2003). Selective upregulation of a single distinctly structured var gene in chondroitin sulphate A-adhering Plasmodium falciparum involved in pregnancy-associated malaria. Molecular microbiology *49*, 179-191.
Salmon, B.L., Oksman, A., and Goldberg, D.E. (2001). Malaria parasite exit from the host erythrocyte: a two-step process requiring extraerythrocytic proteolysis. P Natl Acad Sci USA *98*, 271-276.
Sanders, P.R., Kats, L.M., Drew, D.R., O'Donnell, R.A., O'Neill, M., Maier, A.G., Coppel, R.L., and Crabb, B.S. (2006). A set of glycosylphosphatidyl inositol-anchored membrane proteins of Plasmodium falciparum is refractory to genetic deletion. Infection and Immunity *74*, 4330-4338.
Sargeant, T.J., Marti, M., Caler, E., Carlton, J.M., Simpson, K., Speed, T.P., and Cowman, A.F. (2006). Lineage-specific expansion of proteins exported to erythrocytes in malaria parasites. Genome biology *7*, R12.
Sarthou, J.L., Angel, G., Aribot, G., Rogier, C., Dieye, A., Toure Balde, A., Diatta, B., Seignot, P., and Roussilhon, C. (1997). Prognostic value of anti-Plasmodium falciparum-specific immunoglobulin G3, cytokines, and their soluble receptors in West African patients with severe malaria. Infection and Immunity *65*, 3271-3276.

Schofield, L., Villaquiran, J., Ferreira, A., Schellekens, H., Nussenzweig, R., and Nussenzweig, V. (1987). Gamma interferon, CD8+ T cells and antibodies required for immunity to malaria sporozoites. Nature *330*, 664-666.

Scholander, C., Carlson, J., Kremsner, P.G., and Wahlgren, M. (1998). Extensive immunoglobulin binding of Plasmodium falciparum-infected erythrocytes in a group of children with moderate anemia. Infection and Immunity *66*, 361-363.

Schreiber, N., Brattig, N., Evans, J., Tsiri, A., Horstmann, R.D., May, J., and Klinkert, M.Q. (2006). Cerebral malaria is associated with IgG2 and IgG4 antibody responses to recombinant Plasmodium falciparum RIFIN antigen. Microbes and infection / Institut Pasteur *8*, 1269-1276.

Shi, Y.P., Sayed, U., Qari, S.H., Roberts, J.M., Udhayakumar, V., Oloo, A.J., Hawley, W.A., Kaslow, D.C., Nahlen, B.L., and Lal, A.A. (1996). Natural immune response to the C-terminal 19-kilodalton domain of Plasmodium falciparum merozoite surface protein 1. Infection and Immunity *64*, 2716-2723.

Shulman, C.E., Graham, W.J., Jilo, H., Lowe, B.S., New, L., Obiero, J., Snow, R.W., and Marsh, K. (1996). Malaria is an important cause of anaemia in primigravidae: evidence from a district hospital in coastal Kenya. T Roy Soc Trop Med H *90*, 535-539.

Silamut, K., Phu, N.H., Whitty, C., Turner, G.D., Louwrier, K., Mai, N.T., Simpson, J.A., Hien, T.T., and White, N.J. (1999). A quantitative analysis of the microvascular sequestration of malaria parasites in the human brain. The American journal of pathology *155*, 395-410.

Silverstein, R.L., and Febbraio, M. (2009). CD36, a scavenger receptor involved in immunity, metabolism, angiogenesis, and behavior. Sci Signal *2*, re3.

Silvie, O., Franetich, J.F., Charrin, S., Mueller, M.S., Siau, A., Bodescot, M., Rubinstein, E., Hannoun, L., Charoenvit, Y., Kocken, C.H*., et al.* (2004). A role for apical membrane antigen 1 during invasion of hepatocytes by Plasmodium falciparum sporozoites. The Journal of biological chemistry *279*, 9490-9496.

Sim, B.K., Chitnis, C.E., Wasniowska, K., Hadley, T.J., and Miller, L.H. (1994). Receptor and ligand domains for invasion of erythrocytes by Plasmodium falciparum. Science *264*, 1941-1944.

Sim, B.K., Orlandi, P.A., Haynes, J.D., Klotz, F.W., Carter, J.M., Camus, D., Zegans, M.E., and Chulay, J.D. (1990). Primary structure of the 175K Plasmodium falciparum erythrocyte binding antigen and identification of a peptide which elicits antibodies that inhibit malaria merozoite invasion. The Journal of cell biology *111*, 1877-1884.

Singh, A.P., Buscaglia, C.A., Wang, Q., Levay, A., Nussenzweig, D.R., Walker, J.R., Winzeler, E.A., Fujii, H., Fontoura, B.M., and Nussenzweig, V. (2007). Plasmodium circumsporozoite protein promotes the development of the liver stages of the parasite. Cell *131*, 492-504.

Singh, S., Alam, M.M., Pal-Bhowmick, I., Brzostowski, J.A., and Chitnis, C.E. (2010). Distinct external signals trigger sequential release of apical organelles during erythrocyte invasion by malaria parasites. PLoS pathogens *6*, e1000746.

Sirima, S.B., Nebie, I., Ouedraogo, A., Tiono, A.B., Konate, A.T., Gansane, A., Derme, A.I., Diarra, A., Soulama, I., Cuzzin-Ouattara, N*., et al.* (2007). Safety and immunogenicity of the Plasmodium falciparum merozoite surface protein-3 long synthetic peptide (MSP3-LSP) malaria vaccine in healthy, semi-immune adult males in Burkina Faso, West Africa. Vaccine *25*, 2723-2732.

Smith, J.D., Chitnis, C.E., Craig, A.G., Roberts, D.J., Hudson-Taylor, D.E., Peterson, D.S., Pinches, R., Newbold, C.I., and Miller, L.H. (1995). Switches in expression of Plasmodium falciparum var genes correlate with changes in antigenic and cytoadherent phenotypes of infected erythrocytes. Cell *82*, 101-110.

Smith, T., Beck, H.P., Kitua, A., Mwankusye, S., Felger, I., Fraser-Hurt, N., Irion, A., Alonso, P., Teuscher, T., and Tanner, M. (1999). Age dependence of the multiplicity of Plasmodium falciparum infections and of other malariological indices in an area of high endemicity. T Roy Soc Trop Med H *93 Suppl 1*, 15-20.

Smith, T.G., Lourenco, P., Carter, R., Walliker, D., and Ranford-Cartwright, L.C. (2000). Commitment to sexual differentiation in the human malaria parasite, Plasmodium falciparum. Parasitology *121 (Pt 2)*, 127-133.

Smythe, J.A., Coppel, R.L., Brown, G.V., Ramasamy, R., Kemp, D.J., and Anders, R.F. (1988). Identification of two integral membrane proteins of Plasmodium falciparum. P Natl Acad Sci USA *85*, 5195-5199.

Smythe, J.A., Peterson, M.G., Coppel, R.L., Saul, A.J., Kemp, D.J., and Anders, R.F. (1990). Structural diversity in the 45-kilodalton merozoite surface antigen of Plasmodium falciparum. Mol Biochem Parasit *39*, 227-234.

Snewin, V.A., Herrera, M., Sanchez, G., Scherf, A., Langsley, G., and Herrera, S. (1991). Polymorphism of the alleles of the merozoite surface antigens MSA1 and MSA2 in Plasmodium falciparum wild isolates from Colombia. Mol Biochem Parasit *49*, 265-275.

Snounou, G. (2002). Genotyping of Plasmodium spp. Nested PCR. Methods in molecular medicine *72*, 103-116.

Snow, R.W., Nahlen, B., Palmer, A., Donnelly, C.A., Gupta, S., and Marsh, K. (1998). Risk of severe malaria among African infants: direct evidence of clinical protection during early infancy. The Journal of infectious diseases *177*, 819-822.

Spadafora, C., Awandare, G.A., Kopydlowski, K.M., Czege, J., Moch, J.K., Finberg, R.W., Tsokos, G.C., and Stoute, J.A. (2010). Complement receptor 1 is a sialic acid-independent erythrocyte receptor of Plasmodium falciparum. PLoS pathogens *6*, e1000968.

Spycher, C., Rug, M., Pachlatko, E., Hanssen, E., Ferguson, D., Cowman, A.F., Tilley, L., and Beck, H.P. (2008). The Maurer's cleft protein MAHRP1 is essential for trafficking of PfEMP1 to the surface of Plasmodium falciparum-infected erythrocytes. Molecular microbiology *68*, 1300-1314.

Srinivasan, P., Beatty, W.L., Diouf, A., Herrera, R., Ambroggio, X., Moch, J.K., Tyler, J.S., Narum, D.L., Pierce, S.K., Boothroyd, J.C.*, et al.* (2011). Binding of Plasmodium merozoite proteins RON2 and AMA1 triggers commitment to invasion. P Natl Acad Sci USA *108*, 13275-13280.

Stafford, W.H., Stockley, R.W., Ludbrook, S.B., and Holder, A.A. (1996). Isolation, expression and characterization of the gene for an ADP-ribosylation factor from the human malaria parasite, Plasmodium falciparum. European journal of biochemistry / FEBS *242*, 104-113.

Stanisic, D.I., Richards, J.S., McCallum, F.J., Michon, P., King, C.L., Schoepflin, S., Gilson, P.R., Murphy, V.J., Anders, R.F., Mueller, I.*, et al.* (2009). Immunoglobulin G subclass-specific responses against Plasmodium falciparum merozoite antigens are associated with control of parasitemia and protection from symptomatic illness. Infection and Immunity *77*, 1165-1174.

Stanley, H.A., Howard, R.F., and Reese, R.T. (1985). Recognition of a Mr 56K glycoprotein on the surface of Plasmodium falciparum merozoites by mouse monoclonal antibodies. Journal of immunology *134*, 3439-3444.

Stoute, J.A., Slaoui, M., Heppner, D.G., Momin, P., Kester, K.E., Desmons, P., Wellde, B.T., Garcon, N., Krzych, U., and Marchand, M. (1997). A preliminary evaluation of a recombinant circumsporozoite protein vaccine against Plasmodium falciparum malaria. RTS,S Malaria Vaccine Evaluation Group. The New England journal of medicine *336*, 86-91.

Stubbs, J., Simpson, K.M., Triglia, T., Plouffe, D., Tonkin, C.J., Duraisingh, M.T., Maier, A.G., Winzeler, E.A., and Cowman, A.F. (2005). Molecular mechanism for switching of P. falciparum invasion pathways into human erythrocytes. Science *309*, 1384-1387.

Su, X.Z., Heatwole, V.M., Wertheimer, S.P., Guinet, F., Herrfeldt, J.A., Peterson, D.S., Ravetch, J.A., and Wellems, T.E. (1995). The large diverse gene family var encodes proteins involved in cytoadherence and antigenic variation of Plasmodium falciparum-infected erythrocytes. Cell *82*, 89-100.

Sultan, A.A., Thathy, V., Frevert, U., Robson, K.J., Crisanti, A., Nussenzweig, V., Nussenzweig, R.S., and Menard, R. (1997). TRAP is necessary for gliding motility and infectivity of plasmodium sporozoites. Cell *90*, 511-522.

Sutherland, C.J. (2001). Stevor transcripts from Plasmodium falciparum gametocytes encode truncated polypeptides. Mol Biochem Parasit *113*, 331-335.

Takala, S., Branch, O., Escalante, A.A., Kariuki, S., Wootton, J., and Lal, A.A. (2002). Evidence for intragenic recombination in Plasmodium falciparum: identification of a novel allele family in block 2 of merozoite surface protein-1: Asembo Bay Area Cohort Project XIV. Mol Biochem Parasit *125*, 163-171.

Talman, A.M., Domarle, O., McKenzie, F.E., Ariey, F., and Robert, V. (2004). Gametocytogenesis: the puberty of Plasmodium falciparum. Malaria J *3*, 24.

Tanabe, K., Mackay, M., Goman, M., and Scaife, J.G. (1987). Allelic dimorphism in a surface antigen gene of the malaria parasite Plasmodium falciparum. Journal of molecular biology *195*, 273-287.

Tarun, A.S., Baer, K., Dumpit, R.F., Gray, S., Lejarcegui, N., Frevert, U., and Kappe, S.H. (2006). Quantitative isolation and in vivo imaging of malaria parasite liver stages. International journal for parasitology *36*, 1283-1293.

Taverne, J., Sheikh, N., de Souza, J.B., Playfair, J.H., Probert, L., and Kollias, G. (1994). Anaemia and resistance to malaria in transgenic mice expressing human tumour necrosis factor. Immunology *82*, 397-403.

Taylor, H.M., Grainger, M., and Holder, A.A. (2002). Variation in the expression of a Plasmodium falciparum protein family implicated in erythrocyte invasion. Infection and Immunity *70*, 5779-5789.

Taylor, R.R., Allen, S.J., Greenwood, B.M., and Riley, E.M. (1998). IgG3 antibodies to Plasmodium falciparum merozoite surface protein 2 (MSP2): increasing prevalence with age and association with clinical immunity to malaria. The American journal of tropical medicine and hygiene *58*, 406-413.

Taylor, W.R., Canon, V., and White, N.J. (2006). Pulmonary manifestations of malaria : recognition and management. Treatments in respiratory medicine *5*, 419-428.

Taylor, W.R., Hanson, J., Turner, G.D., White, N.J., and Dondorp, A.M. (2012). Respiratory manifestations of malaria. Chest *142*, 492-505.

Tham, W.H., Healer, J., and Cowman, A.F. (2012). Erythrocyte and reticulocyte binding-like proteins of Plasmodium falciparum. Trends in parasitology *28*, 23-30.

Tham, W.H., Schmidt, C.Q., Hauhart, R.E., Guariento, M., Tetteh-Quarcoo, P.B., Lopaticki, S., Atkinson, J.P., Barlow, P.N., and Cowman, A.F. (2011). Plasmodium falciparum uses a key functional site in complement receptor type-1 for invasion of human erythrocytes. Blood *118*, 1923-1933.

Tham, W.H., Wilson, D.W., Reiling, L., Chen, L., Beeson, J.G., and Cowman, A.F. (2009). Antibodies to reticulocyte binding protein-like homologue 4 inhibit invasion of Plasmodium falciparum into human erythrocytes. Infection and Immunity *77*, 2427-2435.

Touray, M.G., Warburg, A., Laughinghouse, A., Krettli, A.U., and Miller, L.H. (1992). Developmentally regulated infectivity of malaria sporozoites for mosquito salivary glands and the vertebrate host. The Journal of experimental medicine *175*, 1607-1612.

Toure, F.S., Deloron, P., and Migot-Nabias, F. (2006). Analysis of human antibodies to erythrocyte binding antigen 175 peptide 4 of Plasmodium falciparum. Clinical medicine & research *4*, 1-6.

Toure, F.S., Mavoungou, E., Ndong, J.M., Tshipamba, P., and Deloron, P. (2001). Erythrocyte binding antigen (EBA-175) of Plasmodium falciparum: improved genotype determination by nested polymerase chain reaction. Tropical medicine & international health : TM & IH *6*, 767-769.

Trager, W., and Jensen, J.B. (1976). Human malaria parasites in continuous culture. Science *193*, 673-675.

Trape, J.F., Rogier, C., Konate, L., Diagne, N., Bouganali, H., Canque, B., Legros, F., Badji, A., Ndiaye, G., Ndiaye, P.*, et al.* (1994). The Dielmo project: a longitudinal study of natural malaria infection and the mechanisms of protective immunity in a community living in a holoendemic area of Senegal. The American journal of tropical medicine and hygiene *51*, 123-137.

Treeck, M., Zacherl, S., Herrmann, S., Cabrera, A., Kono, M., Struck, N.S., Engelberg, K., Haase, S., Frischknecht, F., Miura, K.*, et al.* (2009). Functional analysis of the leading malaria vaccine candidate AMA-1 reveals an essential role for the cytoplasmic domain in the invasion process. PLoS pathogens *5*, e1000322.

Treutiger, C.J., Hedlund, I., Helmby, H., Carlson, J., Jepson, A., Twumasi, P., Kwiatkowski, D., Greenwood, B.M., and Wahlgren, M. (1992). Rosette formation in Plasmodium falciparum isolates and anti-rosette activity of sera from Gambians with cerebral or uncomplicated malaria. The American journal of tropical medicine and hygiene *46*, 503-510.

Treutiger, C.J., Scholander, C., Carlson, J., McAdam, K.P., Raynes, J.G., Falksveden, L., and Wahlgren, M. (1999). Rouleaux-forming serum proteins are involved in the rosetting of Plasmodium falciparum-infected erythrocytes. Experimental parasitology *93*, 215-224.

Triglia, T., Duraisingh, M.T., Good, R.T., and Cowman, A.F. (2005). Reticulocyte-binding protein homologue 1 is required for sialic acid-dependent invasion into human erythrocytes by Plasmodium falciparum. Molecular microbiology *55*, 162-174.

Triglia, T., Healer, J., Caruana, S.R., Hodder, A.N., Anders, R.F., Crabb, B.S., and Cowman, A.F. (2000). Apical membrane antigen 1 plays a central role in erythrocyte invasion by Plasmodium species. Molecular microbiology *38*, 706-718.

Triglia, T., Thompson, J.K., and Cowman, A.F. (2001). An EBA175 homologue which is transcribed but not translated in erythrocytic stages of Plasmodium falciparum. Mol Biochem Parasit *116*, 55-63.

Trimnell, A.R., Kraemer, S.M., Mukherjee, S., Phippard, D.J., Janes, J.H., Flamoe, E., Su, X.Z., Awadalla, P., and Smith, J.D. (2006). Global genetic diversity and evolution of var genes associated with placental and severe childhood malaria. Mol Biochem Parasit *148*, 169-180.

Turner, C.M. (1999). Antigenic variation in Trypanosoma brucei infections: an holistic view. Journal of cell science *112 (Pt 19)*, 3187-3192.

Turner, G.D., Morrison, H., Jones, M., Davis, T.M., Looareesuwan, S., Buley, I.D., Gatter, K.C., Newbold, C.I., Pukritayakamee, S., Nagachinta, B*., et al.* (1994). An immunohistochemical study of the pathology of fatal malaria. Evidence for widespread endothelial activation and a potential role for intercellular adhesion molecule-1 in cerebral sequestration. The American journal of pathology *145*, 1057-1069.

Udomsangpetch, R., Aikawa, M., Berzins, K., Wahlgren, M., and Perlmann, P. (1989). Cytoadherence of knobless Plasmodium falciparum-infected erythrocytes and its inhibition by a human monoclonal antibody. Nature *338*, 763-765.

Valecha, N., Pinto, R.G., Turner, G.D., Kumar, A., Rodrigues, S., Dubhashi, N.G., Rodrigues, E., Banaulikar, S.S., Singh, R., Dash, A.P*., et al.* (2009). Histopathology of fatal respiratory distress caused by Plasmodium vivax malaria. The American journal of tropical medicine and hygiene *81*, 758-762.

van Hensbroek, M.B., Palmer, A., Jaffar, S., Schneider, G., and Kwiatkowski, D. (1997). Residual neurologic sequelae after childhood cerebral malaria. The Journal of pediatrics *131*, 125-129.

van Ooij, C., Tamez, P., Bhattacharjee, S., Hiller, N.L., Harrison, T., Liolios, K., Kooij, T., Ramesar, J., Balu, B., Adams, J*., et al.* (2008). The malaria secretome: from algorithms to essential function in blood stage infection. PLoS pathogens *4*, e1000084.

Vaughan, A.M., Wang, R., and Kappe, S.H. (2010). Genetically engineered, attenuated whole-cell vaccine approaches for malaria. Human vaccines *6*, 107-113.

Vik, D.P., and Wong, W.W. (1993). Structure of the gene for the F allele of complement receptor type 1 and sequence of the coding region unique to the S allele. Journal of immunology *151*, 6214-6224.

Vogt, A.M., Barragan, A., Chen, Q., Kironde, F., Spillmann, D., and Wahlgren, M. (2003). Heparan sulfate on endothelial cells mediates the binding of Plasmodium falciparum-infected erythrocytes via the DBL1alpha domain of PfEMP1. Blood *101*, 2405-2411.

Vogt, A.M., Pettersson, F., Moll, K., Jonsson, C., Normark, J., Ribacke, U., Egwang, T.G., Ekre, H.P., Spillmann, D., Chen, Q*., et al.* (2006). Release of sequestered malaria parasites upon injection of a glycosaminoglycan. PLoS pathogens *2*, e100.

Voss, T.S., Healer, J., Marty, A.J., Duffy, M.F., Thompson, J.K., Beeson, J.G., Reeder, J.C., Crabb, B.S., and Cowman, A.F. (2006). A var gene promoter controls allelic exclusion of virulence genes in Plasmodium falciparum malaria. Nature *439*, 1004-1008.

Wahlgren, M., Carlson, J., Helmby, H., Hedlund, I., and Treutiger, C.J. (1992). Molecular mechanisms and biological importance of Plasmodium falciparum erythrocyte rosetting. Mem I Oswaldo Cruz *87 Suppl 3*, 323-329.

Walker, O., Salako, L.A., Sowunmi, A., Thomas, J.O., Sodeine, O., and Bondi, F.S. (1992). Prognostic risk factors and post mortem findings in cerebral malaria in children. T Roy Soc Trop Med H *86*, 491-493.

Waller, K.L., Cooke, B.M., Nunomura, W., Mohandas, N., and Coppel, R.L. (1999). Mapping the binding domains involved in the interaction between the Plasmodium falciparum knob-associated histidine-rich protein (KAHRP) and the cytoadherence ligand P. falciparum erythrocyte membrane protein 1 (PfEMP1). The Journal of biological chemistry *274*, 23808-23813.

Waller, R.F., Reed, M.B., Cowman, A.F., and McFadden, G.I. (2000). Protein trafficking to the plastid of Plasmodium falciparum is via the secretory pathway. The EMBO journal *19*, 1794-1802.

Waters, A.P., Thomas, A.W., Deans, J.A., Mitchell, G.H., Hudson, D.E., Miller, L.H., McCutchan, T.F., and Cohen, S. (1990). A merozoite receptor protein from Plasmodium knowlesi is highly conserved and distributed throughout Plasmodium. The Journal of biological chemistry *265*, 17974-17979.

Weedall, G.D., and Conway, D.J. (2010). Detecting signatures of balancing selection to identify targets of anti-parasite immunity. Trends in parasitology *26*, 363-369.

White, N.J. (2008). Plasmodium knowlesi: the fifth human malaria parasite. Clinical infectious diseases : an official publication of the Infectious Diseases Society of America *46*, 172-173.

WHO (2000a). Severe falciparum malaria. Trans R Soc Trop Med Hyg *94*, 1-90.

WHO (2000b). Severe falciparum malaria, World Health Organization, Communicable Diseases Cluster. Trans R Soc Trop Med Hyg *94 SUPPL*, S1-S90.

WHO (2010). Guidelines for the treatment of malaria. Second edition.

WHO (2011). World Malaria report 2011.

WHO (2012). World malaria report 2012.

Wickham, M.E., Rug, M., Ralph, S.A., Klonis, N., McFadden, G.I., Tilley, L., and Cowman, A.F. (2001). Trafficking and assembly of the cytoadherence complex in Plasmodium falciparum-infected human erythrocytes. The EMBO journal *20*, 5636-5649.

Wickramasinghe, S.N., and Abdalla, S.H. (2000). Blood and bone marrow changes in malaria. Baillieres Best Pract Res Clin Haematol *13*, 277-299.

Williams, T.N. (2006). Red blood cell defects and malaria. Mol Biochem Parasit *149*, 121-127.

Williams, T.N., Mwangi, T.W., Roberts, D.J., Alexander, N.D., Weatherall, D.J., Wambua, S., Kortok, M., Snow, R.W., and Marsh, K. (2005a). An immune basis for malaria protection by the sickle cell trait. PLoS medicine *2*, e128.

Williams, T.N., Mwangi, T.W., Wambua, S., Alexander, N.D., Kortok, M., Snow, R.W., and Marsh, K. (2005b). Sickle cell trait and the risk of Plasmodium falciparum malaria and other childhood diseases. The Journal of infectious diseases *192*, 178-186.

Williams, T.N., Wambua, S., Uyoga, S., Macharia, A., Mwacharo, J.K., Newton, C.R., and Maitland, K. (2005c). Both heterozygous and homozygous alpha+ thalassemias protect against severe and fatal Plasmodium falciparum malaria on the coast of Kenya. Blood *106*, 368-371.

Wilson, D.W., Fowkes, F.J., Gilson, P.R., Elliott, S.R., Tavul, L., Michon, P., Dabod, E., Siba, P.M., Mueller, I., Crabb, B.S.*, et al.* (2011). Quantifying the importance of MSP1-19 as a target of growth-inhibitory and protective antibodies against Plasmodium falciparum in humans. PLoS One *6*, e27705.

Wilson, J.G., Murphy, E.E., Wong, W.W., Klickstein, L.B., Weis, J.H., and Fearon, D.T. (1986). Identification of a restriction fragment length polymorphism by a CR1 cDNA that correlates with the number of CR1 on erythrocytes. The Journal of experimental medicine *164*, 50-59.

Winter, G., Kawai, S., Haeggstrom, M., Kaneko, O., von Euler, A., Kawazu, S., Palm, D., Fernandez, V., and Wahlgren, M. (2005). SURFIN is a polymorphic antigen expressed on Plasmodium falciparum merozoites and infected erythrocytes. The Journal of experimental medicine *201*, 1853-1863.

Wu, Y., Ellis, R.D., Shaffer, D., Fontes, E., Malkin, E.M., Mahanty, S., Fay, M.P., Narum, D., Rausch, K., Miles, A.P.*, et al.* (2008). Phase 1 trial of malaria transmission blocking vaccine candidates Pfs25 and Pvs25 formulated with montanide ISA 51. PLoS One *3*, e2636.

Ye, J., Bromage, E.S., and Kaattari, S.L. (2010). The strength of B cell interaction with antigen determines the degree of IgM polymerization. Journal of immunology *184*, 844-850.

Yeka, A., Banek, K., Bakyaita, N., Staedke, S.G., Kamya, M.R., Talisuna, A., Kironde, F., Nsobya, S.L., Kilian, A., Slater, M., *et al.* (2005). Artemisinin versus nonartemisinin combination therapy for uncomplicated malaria: randomized clinical trials from four sites in Uganda. PLoS medicine *2*, e190.