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**Naturally Acquired Immunity to *Plasmodium*  
*falciparum* Malaria: Antibody Responses and  
Immunological Memory**

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To my mother *Dr. Jennifer Jepkurui*



## ABSTRACT

*Plasmodium falciparum* malaria is a significant public health concern particularly in Sub-Saharan Africa. Effective malaria vaccines will contribute significantly towards controlling the disease but their development is hampered by the incomplete understanding of immunity to malaria. Whereas naturally acquired immunity is known to have an important antibody mediated component, the targets and functional correlates as well longevity of these responses are largely unknown and merit further understanding. The studies presented in this thesis, investigate several aspects of naturally acquired immunity to some of the major merozoite vaccine candidate antigens.

In longitudinally monitored children in Tanzania, antibody responses against seven merozoite surface antigens were investigated in relation to the genetic diversity of *P. falciparum* infections, as determined by genotyping of one of the merozoite surface protein genes. The breadth of anti-merozoite antibody responses was positively correlated with the number of concurrent *P. falciparum* clones in asymptomatic children. Further, broad antibody responses and genetically diverse infections, in combination, were more strongly associated with protection against malaria than they were individually suggesting that multicomponent malaria vaccines mimicking naturally acquired immunity should ideally induce antibody responses that can be boosted by natural infections.

The inhibitory activity of naturally acquired antibodies on the *in vitro* growth of *P. falciparum* in relation to merozoite invasion phenotype was investigated in a case-control study in Tanzanian children. The growth-inhibitory activities (GIA) of plasma were different when tested on different parasite lines. The association between GIA and protection against clinical malaria was also parasite line-dependent thus emphasizing the importance of invasion phenotypes as well as the need to consider the choice of parasite lines in the use of GIA as a correlate of protection against clinical malaria in epidemiological and vaccine studies.

Within a longitudinally monitored population in Kenya, temporal dynamics of anti-merozoite antibody responses were investigated in children with different susceptibilities to malaria. Overall, antibody levels were similar in children experiencing multiple episodes or only single episodes suggesting that differences in disease susceptibility are not attributable to differences in the acquisition of anti-merozoite antibody responses, and may be explained by other factors, such as differences in the intensity of exposure to the parasite in this setting of low-moderate malaria transmission.

To investigate the longevity of immune responses induced by natural *P. falciparum* infections, circulating merozoite antigen-specific antibodies and memory B-cells (MBCs) were studied in travelers who had been diagnosed and treated for malaria in Stockholm 1-16 years previously. *P. falciparum*-specific MBCs, but not antibodies, were found to have been maintained for up to 16 years without re-exposure to the parasite.

In conclusion, single natural *P. falciparum* infections induce long-lived memory-B cell responses to merozoite antigens, however, broad and protective antibody responses require repeated exposure and preferably persistent genetically diverse infections to confer clinical immunity to malaria. Taken together, these studies advance the understanding of naturally acquired immunity to malaria and have important implications for the development of malaria vaccines.

## LIST OF PUBLICATIONS

- I. **Josea Rono**, Faith H. A. Osier, Daniel Olsson, Scott Montgomery, Leah Mhoja, Ingegerd Rooth, Kevin Marsh and Anna Färnert

Breadth of anti-merozoite antibody responses is associated with the genetic diversity of symptomatic *Plasmodium falciparum* infections and protection against clinical malaria

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- II. **Josea Rono**, Anna Färnert, Daniel Olsson, Faith Osier, Ingegerd Rooth, Kristina E. M. Persson.

*Plasmodium falciparum* line-dependent association of *in vitro* growth-inhibitory activity and risk of malaria

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- III. **Josea Rono**, Anna Färnert, John Ojal, George Nyangweso, Linda Murungi, Gathoni Kamuyu, Juliana Wambua, Ally Olotu, Kevin Marsh, and Faith H. A. Osier

Five-year Temporal Dynamics of Naturally Acquired Antibodies to *Plasmodium falciparum* Merozoite Antigens in Children experiencing Multiple episodes of Malaria

*Manuscript*.

- IV. Francis M Ndungu, Klara Lundblom, **Josea Rono**, Joseph Illingworth, Sara Eriksson and Anna Färnert

Long-Lived *Plasmodium falciparum*-Specific Memory B cells in Naturally Exposed Swedish Travelers

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# TABLE OF CONTENTS

1	INTRODUCTION .....	1
1.1	The malaria parasite.....	1
1.2	The epidemiology of <i>Plasmodium falciparum</i> malaria.....	1
1.3	The life cycle and pathogenesis of <i>Plasmodium falciparum</i> .....	3
1.4	Clinical presentation of <i>Plasmodium falciparum</i> malaria.....	7
1.5	Naturally acquired immunity to malaria .....	8
1.6	Malaria control interventions .....	9
1.7	Malaria vaccines .....	10
1.8	<i>Plasmodium falciparum</i> merozoite antigens.....	14
1.9	Antibody responses to the <i>Plasmodium falciparum</i> merozoite surface antigens .....	16
1.10	Genetic diversity of <i>Plasmodium falciparum</i> .....	18
1.11	Immunological memory to <i>Plasmodium falciparum</i> merozoite antigens	21
2	AIM OF THE THESIS .....	25
3	MATERIALS AND METHODS .....	26
3.1	STUDY POPULATIONS .....	26
3.1.1	Tanzania (Study I and II).....	26
3.1.2	Kenya (Study III) .....	26
3.1.3	Travelers diagnosed with <i>Plasmodium falciparum</i> malaria in Sweden (Study IV).....	27
3.2	ETHICAL CONSIDERATIONS.....	27
3.3	DETERMINATION OF THE GENETIC DIVERSITY OF ASYMPTOMATIC <i>PLASMODIUM FALCIPARUM</i> INFECTIONS....	27
3.4	QUANTIFICATION OF ANTI- <i>PLASMODIUM FALCIPARUM</i> ANTIBODIES .....	28
3.4.1	Recombinant merozoite antigens .....	28
3.4.2	Quantification of merozoite antigen-specific antibody responses using a multiplex bead-based assay.....	28
3.4.3	Quantification of antibody responses to <i>Plasmodium falciparum</i> schizont extracts .....	30
3.5	QUANTIFICATION OF THE INHIBITORY ACTIVITY OF SERA ON THE <i>IN VITRO</i> GROWTH OF <i>PLASMODIUM FALCIPARUM</i> ..	30
3.6	PHENOTYPING MEMORY B-CELL SUBSETS.....	31
3.7	QUANTIFICATION OF <i>PLASMODIUM FALCIPARUM</i> -SPECIFIC MEMORY B-CELLS .....	31
3.8	STATISTICAL ANALYSES .....	32
4	RESULTS .....	34
4.1	STUDY I Breadth of anti-merozoite antibody responses is associated with the genetic diversity of asymptomatic <i>Plasmodium falciparum</i> infections and protection against clinical malaria.....	34
4.2	STUDY II <i>Plasmodium falciparum</i> line-dependent association of <i>in vitro</i> growth-inhibitory activity and risk of malaria .....	35

4.3	STUDY III Five-year temporal dynamics of naturally acquired antibodies to <i>Plasmodium falciparum</i> merozoite antigens in children experiencing multiple episodes of malaria .....	36
4.4	STUDY IV Long-lived <i>Plasmodium falciparum</i> -specific memory B cells in naturally exposed Swedish travelers.....	37
5	DISCUSSION.....	38
6	CONCLUSIONS AND FUTURE PERSPECTIVES .....	43
7	ACKNOWLEDGEMENTS .....	45
8	REFERENCES .....	48

## LIST OF ABBREVIATIONS

AMA	Apical membrane antigen 1
CI	Confidence interval
DHE	Dihydroethidium
EBA	Erythrocyte binding antigen
EDTA	Ethylenediaminetetraacetic acid
EIR	Entomological inoculation rate
ELISA	Enzyme-linked immunosorbent assay
Etbr	Ethidium bromide
GIA	Growth-inhibitory activity
Hb	Haemoglobin
Ig	Immunoglobulin
IQR	Interquartile range
LLPCs	Long-lived plasma cells
MBCs	Memory B-cells
MSP	Merozoite surface protein
PCR	Polymerase chain reaction
PfRH	<i>Plasmodium falciparum</i> reticulocyte binding homolog
PfSPZ	<i>Plasmodium falciparum</i> sporozoites
RBC	Red blood cell
SEM	Standard error of mean
SLPCs	Short-lived plasma cells
TBV	Transmission blocking vaccines
WHO	World Health Organisation



# 1 INTRODUCTION

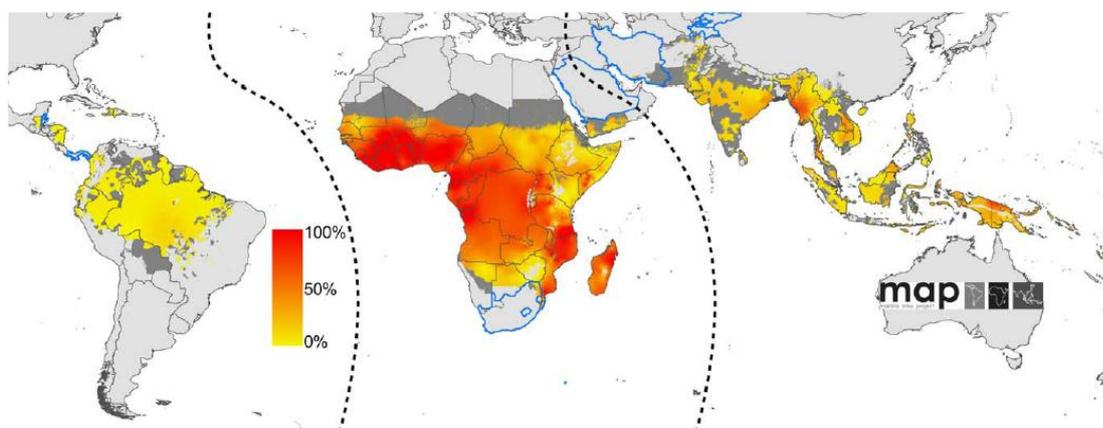
## 1.1 THE MALARIA PARASITE

Malaria is a major disease of humans caused by protozoan parasites belonging to the genus *Plasmodium* within the apicomplexa phylum. Four *Plasmodium* species, *P. falciparum*, *P. vivax*, *P. ovale*, and *P. malariae*, have long been known to be infective to man. *P. knowlesi* which was previously known to infect macaques, has been shown to also infect humans (reviewed in Singh *et al.* 2013). This thesis focuses on *P. falciparum* to which most of the morbidity and mortality of malaria is attributable (Snow *et al.* 2005).

## 1.2 THE EPIDEMIOLOGY OF *PLASMODIUM FALCIPARUM* MALARIA

*P. falciparum* malaria is a significant public health concern globally and in sub-Saharan Africa (SSA) in particular. According to the World Health Organisation (WHO)'s World Malaria Report of 2012, approximately 90% of the estimated 219 (range 154-289) million cases of malaria in 2010 were attributable to *P. falciparum* (WHO 2012). In that report, WHO estimates that there were 660,000 (range 490,000 – 836,000) deaths due to malaria in the same year (WHO 2012). However, a recent independent systematic review reports that global malaria-attributable mortality in 2010 was as high as 1,113,000 (range 848,000 – 1,591,000) (Murray *et al.* 2012). Despite the inconsistencies in the latest global estimates of malaria-attributable mortality, there is consensus that, the bulk of malaria-attributable malaria is in sub-Saharan Africa (Figure 1) and that, on a global scale, malaria-attributable mortality has declined over the past decade (Murray *et al.* 2012; WHO 2012). This decline in malaria disease burden has however not been homogenous. For instance, in Uganda, assessment of paediatric admission data between 1999 and 2009 showed that malaria morbidity in 4 out of 5 government-sponsored hospitals increased by between 47% and 350% over this period (Okiro *et al.* 2011). Further, the incidence of malaria in some regions of Uganda remains as high as 5.3 episodes per person per year (Jagannathan *et al.* 2012). Similarly to Uganda, malaria incidence in Malawi seems to be increasing (Okiro *et al.* 2013). Heterogeneity in the temporal trends of malaria disease burden is also evident at

country level. In Kenya, for instance, whereas hospital admissions have decreased in some regions, they have increased significantly in others (Okiro *et al.* 2009).



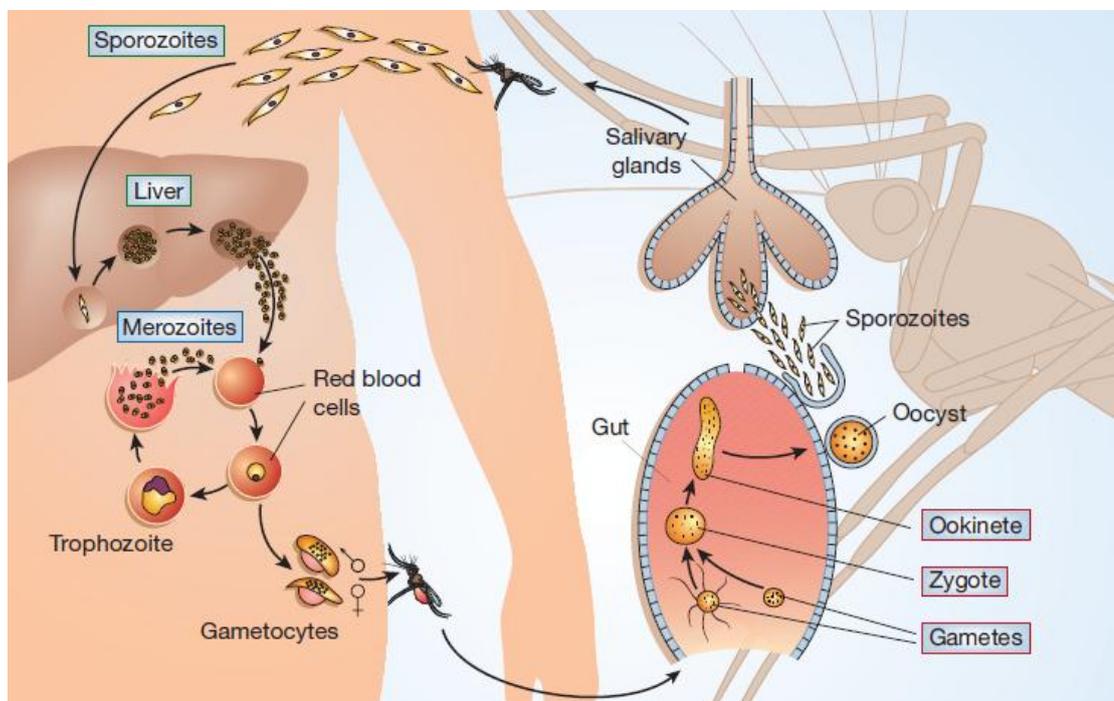
**Figure 1.** Global limits and endemicity of *Plasmodium falciparum* as estimated in 2007. (Adopted from Hay *et al.* 2010).

Similar to malaria morbidity, the reduction in *P. falciparum* transmission intensity has not been evident in all parts of Africa (reviewed in O'Meara *et al.* 2010). For instance, transmission intensity has remained stable or even increased in some parts of the continent (Himeidan *et al.* 2007; Proietti *et al.* 2011).

Heterogeneity in risk of *P. falciparum* malaria is evident even in human populations that reside in close proximity to each other and has long been described as a feature of the micro-epidemiology of malaria (Greenwood *et al.* 1987; Snow *et al.* 1988; Greenwood 1989). This phenomenon, which was initially described in The Gambia, West Africa, has in recent years been shown to be common across many regions of Africa such as in; Kenya (Brooker *et al.* 2004; Ernst *et al.* 2006; Mwangi *et al.* 2008; Bejon *et al.* 2010), Uganda (Clark *et al.* 2008), Tanzania (Bousema *et al.* 2010), Mali (Gaudart *et al.* 2006), Ghana (Kreuels *et al.* 2008), Senegal (Trape *et al.* 2002), Ethiopia (Yeshiwondim *et al.* 2009) and Sudan (Creasey *et al.* 2004). Foci of high malaria incidence (also referred to as “hotspots”) within these populations may provide attractive opportunities of targeted control measures (Dye *et al.* 1986; Woolhouse *et al.* 1997).

### 1.3 THE LIFE CYCLE AND PATHOGENESIS OF *PLASMODIUM FALCIPARUM*

The life cycle of *P. falciparum* involves both sexual and asexual stages in both the mosquito vector and human host (Figure 2). As with all other *Plasmodium* species, human infections caused by *P. falciparum* begin with the bite of an infected female *anopheles* mosquito. Sporozoite forms of the parasite, most of which are injected into the dermis and not directly into blood vessels (Sidjanski *et al.* 1997; Medica *et al.* 2005), glide through the dermis, penetrate blood vessels and enter the circulatory system through which they are taken to the liver (Amino *et al.* 2006; Amino *et al.* 2006). In the liver, sporozoites traverse between Kupffer cells, invade hepatocytes and reside within parasitophorous vacuoles (Mota *et al.* 2001; Mota *et al.* 2001; Prudencio *et al.* 2011). Each sporozoite differentiates and replicates asexually to give rise to thousands of merozoite forms of the parasite. Membrane bound merozoites that contain the exoerythrocytic merozoites then bud off the hepatocytes into the liver sinusoids (Sturm *et al.* 2006) which later rupture and release merozoites into the blood stream.

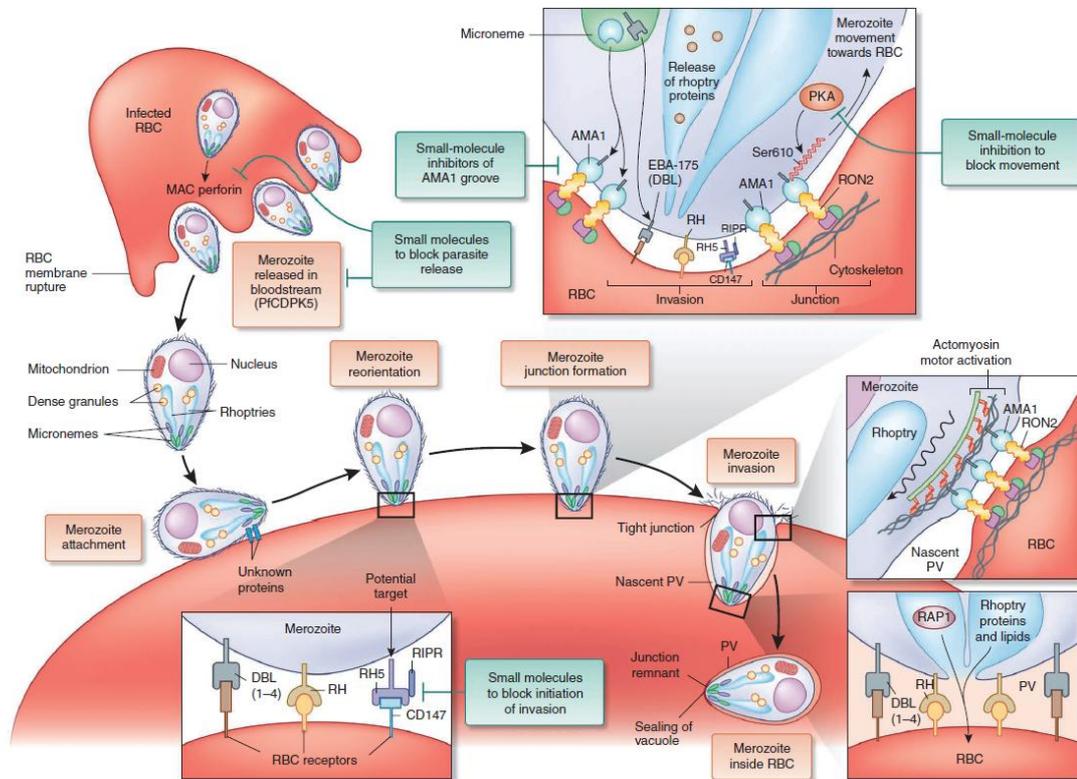


**Figure 2.** The life cycle of *Plasmodium falciparum*. (Adopted from Ménard *et al.*, 2005 and published with permission from Nature Publishing Group).

The release of merozoites into blood exposes them to a milieu in which potassium concentrations are low relative to the concentrations in erythrocytes. This change in potassium concentrations appears to be the necessary extracellular signal that triggers the release of calcium from intracellular stores within the merozoite which in turn drives the secretion of invasins and adhesins from the micronemes onto the merozoite surface (Singh *et al.* 2010). This precedes a multistep erythrocyte invasion process mediated by multiple extracellular recognition events (Figure 3). On encountering an erythrocyte, the merozoite attaches to the erythrocyte surface via low-affinity interactions (Dvorak *et al.* 1975) that are likely to be mediated by merozoite surface proteins such as; MSP-1 (O'Donnell *et al.* 2000; O'Donnell *et al.* 2001), PfMSPDBL-1 (Sakamoto *et al.* 2012), PfMSPDBL-2 (Hodder *et al.* 2012) and proteins of the 6-cys protein family (Ishino *et al.* 2005). The merozoite then undergoes apical re-orientation mediated by the apical membrane antigen 1 (AMA-1) (Mitchell *et al.* 2004). Irreversible attachment to the erythrocyte and commitment to invasion then follows mediated by a series of extracellular recognition events between erythrocyte receptors and ligands on the merozoite which are broadly divided into erythrocyte binding antigens (EBA); EBA140, EBA 175, EBA181 and *P. falciparum* reticulocyte binding homolog (PfRh) proteins; PfRh1, PfRh2a, PfRh2b, PfRh4 and PfRh5 (reviewed in Cowman *et al.* 2012). Apart from PfRH5 (Crosnier *et al.* 2011), *P. falciparum* merozoites are known to vary the expression and use of the other merozoite ligands in erythrocyte invasion such that the parasite is able to utilize multiple redundant invasion pathways (Baum *et al.* 2003; Stubbs *et al.* 2005; Nery *et al.* 2006; Persson *et al.* 2008). Based on this, invasion phenotypes of *P. falciparum* lines can be broadly classified as; sialic acid-dependent (characterised by limited invasion of neuraminidase-treated erythrocytes and mediated by EBAs and PfRh1), or sialic acid-independent invasion, characterised by efficient invasion of neuraminidase treated erythrocytes and mediated by PfRh2b and PfRh4 (reviewed in Cowman *et al.* 2012).

After apical interaction has occurred, there is release of proteins from the micronemes and rhoptry organelles. The RON complex, released from the rhoptry neck, attaches to the erythrocyte membrane with RON2 acting as an anchor (Besteiro *et al.* 2011). AMA-1 then complexes with RON2 thus forming a junction between the merozoite and the erythrocyte (Riglar *et al.* 2011). The inhibition of merozoite invasion in genetically-distinct *P. falciparum* lines by a small molecule that prevents the interaction between AMA-1 and RON2 suggests that the AMA-1-RON2 complex is crucial for

merozoite invasion (Srinivasan *et al.* 2013). The subsequent entry of the merozoite into the erythrocyte involves an ATP-dependent actin-myosin motor (Baum *et al.* 2006).



**Figure 3.** Merozoite invasion into the erythrocyte.

(Adopted from Miller *et al.*, 2013 and published with permission from Nature Publishing Group).

Once inside the erythrocyte the parasite remodels the host cell by increasing permeation across the erythrocytes plasma membrane for uptake of nutrients (Ginsburg 1994; Desai *et al.* 2000; Kirk 2001) and expressing parasite-derived adhesins on knob-like electron-dense protrusions on the erythrocyte surface (Luse *et al.* 1971; Aikawa 1988). One of the best characterised proteins expressed at these knob-like protrusions is *P. falciparum* erythrocyte membrane protein 1, which mediates the binding of infected erythrocytes to the endothelium via several receptors (Baruch 1999; Newbold *et al.* 1999; Chen *et al.* 2000). Binding of infected erythrocytes to the endothelium is thought to prevent destruction of the parasite in the spleen as non-adherent infected erythrocytes are rapidly cleared (Langreth *et al.* 1985). Endothelial binding mediates sequestration of infected erythrocytes in various organs which leads to pathology (reviewed in Miller *et al.* 2002). For instance, sequestration in the brain and placenta are thought to cause cerebral malaria (Newbold *et al.* 1999) and placental malaria (Fried *et al.* 1996)

respectively. Sequestration in the lungs and in other organs is thought to lead to reduced oxygen delivery to tissues which contributes to lactic acidosis (English *et al.* 1997), the predominant form of metabolic acidosis observed in malaria (English *et al.* 1996). Metabolic acidosis leads directly to the syndrome of respiratory distress (Taylor *et al.* 1993).

Parallel to the parasite-driven host cell remodelling events described above, the parasite undergoes a 48-hour maturation process inside the erythrocyte by first differentiating into a ring-stage trophozoite, then into a pigmented trophozoite and finally undergoing asexual replication into a schizont containing several merozoites. The merozoites are released after erythrocyte rupture and can re-invade another erythrocyte to resume another round of asexual reproduction. In the non-immune human host, this leads to an exponential increase in parasitaemia and the onset of the clinical symptoms of malaria can begin within seven days of sporozoite inoculation into the human host (Fairley 1947; Simpson *et al.* 2002). The destruction of erythrocytes by merozoite invasion and schizont rupture is thought to contribute to malarial anaemia (reviewed in Miller *et al.* 2002).

A subset of parasites in the erythrocytic developmental stage differentiate into male and female gametocytes which can remain in circulation for 10-15 days and can be ingested by a feeding female *Anopheles* mosquito (Day *et al.* 1998). In the mosquito, gametocytes differentiate into male and female gametes and fuse to form diploid zygotes which develop into motile and invasive ookinetes. Ookinetes penetrate the mosquito gut wall and encyst into oocysts in which they replicate into thousands of sporozoites. Upon rupture of the oocyst, sporozoites migrate to the salivary glands and can infect man when the mosquito takes a blood meal (Touray *et al.* 1992).

There is growing evidence that part of the pathology associated with *P. falciparum* malaria is immune-mediated (Artavanis-Tsakonas *et al.* 2003). Severe anaemia and cerebral malaria, for instance, are associated with high circulating titres of tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) (Grau *et al.* 1989; Kwiatkowski *et al.* 1990). Studies on the *in vitro* release of pro-inflammatory molecules from macrophages using subcellular parasite compartments have showed that the release of both TNF- $\alpha$  (Schofield *et al.* 1993) and nitric oxide (Tachado *et al.* 1996) is induced by

glycosylphosphatidylinositol (GPI) (an anchor of several proteins found on the merozoite surface including MSP-1 and MSP-2 (Gilson *et al.* 2006)).

#### **1.4 CLINICAL PRESENTATION OF *PLASMODIUM FALCIPARUM* MALARIA**

The outcome of *P. falciparum* infections range from benign asymptomatic infections to death (reviewed in Miller *et al.* 2002) and depends on several factors such as age, immunity, host genetic composition, co-morbidities and parasite phenotype. *P. falciparum* malaria can present clinically with a wide spectrum of features ranging from non-specific flu-like symptoms, to overt and life-threatening clinical conditions. *P. falciparum* infections initially present as flu-like illness with symptoms such as fever, chills, malaise, muscle aches, nausea and headache. The febrile episodes are usually sporadic and do not normally mirror the synchronous erythrocytic development of the parasite. Similarly, the chills are usually intermittent and of sudden onset.

*P. falciparum* infections can also result in severe life-threatening malaria which presents mainly as impaired consciousness, respiratory distress, severe anaemia and multi-organ failure (Marsh *et al.* 1995; WHO 2006; Dondorp *et al.* 2008). In the absence of treatment, severe malaria is almost always fatal (WHO 2010). Cerebral malaria is characterised by unarousable coma and is associated with a mortality rate of 15-20% (reviewed in Mishra *et al.* 2009). Cerebral malaria is more common in areas of low compared to high malaria transmission intensities (Snow *et al.* 1997; Reyburn *et al.* 2005). In children with cerebral malaria, coma is often preceded by seizures whereas in adults seizures are only occasionally observed (reviewed in Mishra *et al.* 2009). Cerebral malaria is associated with neurological sequelae characterised by hemiparesis, cortical blindness and cognitive impairment (Newton *et al.* 1998). Another form of severe malaria is pregnancy-associated malaria which causes maternal anaemia, low birth weight and premature delivery (reviewed in Kane *et al.* 2011). Respiratory distress is characterised by dyspnea, cough and may progress to life-threatening hypoxia. Mortality due to respiratory distress in children has been shown to be particularly high in children who also present with impaired consciousness (Marsh *et al.* 1995). The pathogenesis of respiratory distress is not fully understood. In children, respiratory distress is thought to represent a compensatory mechanism for metabolic acidosis (Marsh *et al.* 1995). Severe anaemia, defined as haemoglobin level lower than 5 g/dl and is more common in areas of high compared to low malaria

transmission intensity and often manifests in young children and pregnant mothers (reviewed in Lamikanra *et al.* 2007). It has been suggested that anaemia is a consequence of the destruction of parasitized and unparasitized erythrocytes and suppression or dysregulation of erythropoiesis (Clark *et al.* 1988; Jakeman *et al.* 1999).

## 1.5 NATURALLY ACQUIRED IMMUNITY TO MALARIA

Individuals living in malaria-endemic areas naturally develop immunity to clinical malaria over several years (reviewed in Marsh *et al.* 2006). Under intense *P. falciparum* transmission, immunity to severe disease and death is acquired within the first five years of life whilst immunity to mild disease is acquired by late adolescence (reviewed in Marsh *et al.* 2006) and seems to wane in the absence of ongoing *P. falciparum* exposure (Colbourne 1955). Studies conducted in Indonesia that compared immune responses between lifelong residents of *P. falciparum*-endemic islands of Indonesia and transmigrants following their re-settlement from non-*P. falciparum*-endemic islands in Java (Baird *et al.* 1991; Hudson Keenihan *et al.* 2003), suggest that adults naturally acquire immunity to malaria faster than children. These observations imply that the natural acquisition of immunity to malaria is at least partly attributable to intrinsic age-specific host factors. Sterile immunity to infection rarely develops, if at all, even in adults with long term exposure to intense *P. falciparum* transmission (reviewed in Marsh *et al.* 2006). Further, the observation of a high incidence of *P. falciparum* parasitemia in peripheral circulation following drug cure, albeit with partially effective drugs, in adults despite their longstanding prior exposure to the parasite strongly suggests that sterilizing immunity is never fully achieved (Doolan *et al.* 2009). This is in contrast to the sterilizing immunity induced following sporozoite inoculation in experimental human (Hoffman *et al.* 2002; Roestenberg *et al.* 2009; Seder *et al.* 2013) and mouse malaria models (Nussenzweig *et al.* 1967).

Although, naturally acquired immunity to clinical malaria has been shown to have a key antibody-mediated component (Cohen *et al.* 1961; Sabchareon *et al.* 1991), it still remains incompletely understood why the acquisition of antibody-mediated immunity is slow (reviewed in Struik *et al.* 2004; Langhorne *et al.* 2008). On one hand, it has been proposed that the slow acquisition of protective antibodies in endemic areas reflects the need to develop a progressively enlarging panel of antibody specificities that eventually enable recognition of several antigenically diverse infections (Day *et al.* 1991). Indeed, there is extensive genetic diversity in *P. falciparum* antigens (reviewed

in Takala *et al.* 2009) and the parasite clonally varies the antigens it expressed on the surface of parasitized erythrocytes (Scherf *et al.* 2008). On the other hand, there is growing evidence that the long duration required to acquire protective antibodies against malaria may be due to dysregulation of B-cell function by *P. falciparum* infections (reviewed in Portugal *et al.* 2013).

## 1.6 MALARIA CONTROL INTERVENTIONS

According to the World Health Organisation (WHO) the recommended approaches for the control of *P. falciparum* malaria are broadly classified into case management and prevention (WHO 2012). Injectable artesunate and artemisinin-based combination therapies are recommended for the management of severe and uncomplicated malaria respectively. Currently, five artemisinin-based combinations are recommended for use; dihydroartemisinin plus piperazine, artesunate plus sulfadoxine-pyrimethamine, artemether plus lumefantrine, artesunate plus amodiaquine and artesunate plus mefloquine (WHO 2012). The approaches recommended by WHO for prevention of malaria are broadly classified as; (i) vector control by means of indoor residual spraying (IRS), insecticide treated nets (ITN) and larval control, (ii) preventive chemotherapy by means of intermittent preventive therapy (IPT) and (iii) prompt diagnosis and treatment of malaria. IRS involves the spraying of residual insecticides onto the inner surfaces of houses where anopheline mosquitoes tend to rest after taking a blood meal. IRS has been shown to be effective in reducing malaria transmission and *P. falciparum*-attributable mortality provided that most houses and animal shelters (>80%) in targeted communities are treated (WHO 2006). ITNs, which include long-lasting insecticidal nets and conventional untreated nets reduce human – mosquito vector contact. ITNs have been shown to reduce *P. falciparum*-attributable morbidity and mortality in children (D'Alessandro *et al.* 1995). Larval control is only recommended in settings where mosquito breeding sites are few, fixed and easily identifiable such that a high proportion of the breeding sites within the flight range of the vector can be treated (WHO 2012). IPT involves the administration of a full course of an effective antimalarial treatment at specified time points to pregnant women (IPTp), infants (IPTi) or a defined population at risk of malaria, regardless of whether they are parasitaemic, with the aim of reducing the population's malaria burden. In areas of moderate to high malaria transmission, IPT with sulfadoxine-pyrimethamine is recommended for all pregnant women at each scheduled antenatal care visit (WHO

2012). Similarly, it is recommended that infants at risk of *P. falciparum* infection in areas of with moderate to high malaria transmission in sub-Saharan Africa receive 3 doses of sulfadoxine-pyrimethamine alongside their routine immunization programme. IPT administration to pregnant women has been shown to be associated with reduced anaemia in the mother and increased birth weight of the child (Gies *et al.* 2009). Analysis of data from several studies of IPTi has shown that the intervention is associated with a reduction of the incidence of clinical malaria of approximately 20-30% (Aponte *et al.* 2009).

## **1.7 MALARIA VACCINES**

The eradication of small pox by a single efficacious vaccine is a prime example of the huge impact that vaccines can have on global health (Foege 2012). In line with this, the development of an effective vaccine against *P. falciparum* malaria would be a major advance in controlling the disease. Contemporary vaccine development efforts are targeted at the sporozoite, pre-erythrocytic, erythrocytic and sexual stages of the parasite. The sporozoite and pre-erythrocytic stages are attractive targets since vaccines that can prevent their establishment will in turn prevent the development of the infection to erythrocytic stages and thus prevent clinical symptoms and mortality. On the other hand, vaccines directed at erythrocytic stages will either prevent clinical symptoms or prevent blood stage infections altogether. The possibility of preventing malaria transmission by preventing the development of gametocytes drives the interest in vaccines against sexual stages of the parasite.

### **1.7.1 Pre-erythrocytic vaccines**

None of the malaria vaccines developed this far has attained the vaccine efficacy target of 80% set by the World Health Organization malaria vaccine technology roadmap (WHO 2006). The most advanced subunit vaccine candidate is RTS,S whose core consists of the 16 NANP repeats proximal to the C-terminus and the entire adjacent C-terminus of the circumsporozoite surface protein fused to the hepatitis B virus surface antigen (Vekemans *et al.* 2008). Low immunogenicity of the RTS,S core antigen in challenge studies necessitated its formulation with the potent AS01 and AS02 adjuvant systems (Stoute *et al.* 1997). Despite addition of the adjuvant, RTS,S/AS01 conferred protection against controlled human malaria infection in only 22% of vaccinees 5 months after their last vaccine dose (Kester *et*

*al.* 2009). Phase II trials of RTS,S/AS02 and RTS,S/AS01 showed protective efficacies of 30% and 56% against malaria respectively (Alonso *et al.* 2004; Bejon *et al.* 2008). In phase III efficacy trials, RTS,S/AS01 showed 30% and 50% protection against malaria in African infants (Agnandji *et al.* 2012) and children (Agnandji *et al.* 2011) respectively. Further, the protection conferred by RTS,S wanes over time and appears to disappear by 4 years post vaccination (Olotu *et al.* 2013).

Experiments done in the 1960s showed that inoculation of mice with X-irradiated *P. berghei* sporozoites protects them against subsequent infections (Nussenzweig *et al.* 1967). More recently, it has been shown that immunization of humans by the bites of X-irradiated mosquitoes harboring *P. falciparum* sporozoites (PfSPZ) confers protective immunity against pre-erythrocytic parasite stages (Clyde *et al.* 1973; Hoffman *et al.* 2002). Large-scale use of this immunization approach was precluded by the technological difficulty in producing X-irradiation attenuated yet metabolically active sporozoites on an industrial scale to support the development of an injectable vaccine. This difficulty has since been overcome and sporozoites can now be produced on industrial scales that meet regulatory standards (Hoffman *et al.* 2010). Subsequent trials revealed that the protective efficacy of this vaccine was largely dependent on the route of administration. Excellent protection was evident following inoculation of the vaccine by mosquito whereas subcutaneous administration via needle and syringe elicited only low-level immune responses and minimal protection (Epstein *et al.* 2011). The low immunogenicity has been attributed to insufficient PfSPZ antigen presentation following subcutaneous administration of the vaccine (Chakravarty *et al.* 2007). Intravenous administration of this vaccine to non-human primates induced potent and persistent PfSPZ-specific T-cell responses (Epstein *et al.* 2011). A recent trial in humans has shown that repeated intravenous administration of high doses of this vaccine confers protection against controlled malaria infection (Seder *et al.* 2013), suggesting that this vaccination approach may be effective.

### **1.7.2 Asexual blood stage vaccines**

The resolution of fever and marked decrease in *P. falciparum* parasitemia in children following their inoculation with IgG obtained from malaria-immune adults (Cohen *et al.* 1961) has so far been the strongest rationale for the development of asexual blood stage vaccines. The attribution of the malaria pathogenesis, in part, to

blood stage infections as well as studies in humans and murine models that demonstrate that immune responses targeting blood stage antigens can protect against disease or contribute to the control of parasitemia (McGregor 1964; Diggs *et al.* 1975; Mitchell *et al.* 1976) further supports the development of blood-stage vaccines. Merozoite antigens are thought to be represent major protective antibody targets and to be attractive vaccine candidates because of the exposure to host immune responses and their involvement in erythrocyte invasion (reviewed in Richards *et al.* 2009). Several merozoite antigens are currently under development as vaccines. These include proteins that are abundantly expressed on the merozoite surface such as MSP-1 (Ogutu *et al.* 2009), MSP-2 (Genton *et al.* 2002), MSP-3 (Audran *et al.* 2005; Druilhe *et al.* 2005), AMA-1 (Sagara *et al.* 2009) and EBA-175 (El Sahly *et al.* 2010). To date immunization with none of these antigens has, individually, conferred clear clinical protection (Goodman *et al.* 2010). For instance, separate phase II trials of AMA-1 and MSP-1 in African children demonstrated minimal efficacy (Ogutu *et al.* 2009; Sagara *et al.* 2009). The observation that protection against malaria in individuals living in malaria endemic areas increases with increasing breadth of anti-merozoite antibody responses (i.e. the number of antigens to which an individual has high antibody titres) (Osier *et al.* 2008) suggests that vaccines that combine several antigens would confer more protection than single-antigen vaccines. Indeed, several combination vaccines such as; AMA-1 and MSP-1 (Malkin *et al.* 2008), CSP and AMA-1 (Thompson *et al.* 2008) and MSP-1, MSP-2 and ring-infected erythrocyte surface antigen (RESA) (Genton *et al.* 2002) have been developed and tested. There is little evidence, so far, that these combination vaccines offer superior efficacy compared to single-antigen vaccines, although this could be a reflection of the modest efficacy of the individual antigens or the diversity within them. The efficacy of these vaccines may be improved by the combination of several antigens that are individually efficacious (John *et al.* 2005; John *et al.* 2008; Osier *et al.* 2008). Vaccination of macaques suggests that it is feasible to combine RTS,S with MSP-1 and/or AMA-1 in a single vaccine that maintains the immunogenicity of individual components (Pichyangkul *et al.* 2009).

Historically, there has been no systematic approach to the identification and prioritization of blood-stage antigens for vaccine development. Recently, more methodological approaches have capitalized on the completion of the *P. falciparum* genome sequence (Gardner *et al.* 2002) and the development of high throughput *in*

*in vitro* protein expression systems to assemble protein arrays of multiple antigens (Doolan *et al.* 2008; Tsuboi *et al.* 2008; Crompton *et al.* 2010; Trieu *et al.* 2011). These approaches are advantageous since they allow for an unbiased screening of multiple antigens. A disadvantage of *in vitro* protein expression systems is that they express proteins under reducing conditions that do not preserve the disulfide bonds necessary for native folding of extracellular proteins. In light of this, mammalian protein expression systems that express proteins under oxidizing conditions that preserve the native conformation of extracellular proteins have been developed (Bushell *et al.* 2008; Crosnier *et al.* 2011). The ability of these systems to express functionally active full-length proteins such as PfRh5 (Bushell *et al.* 2008; Crosnier *et al.* 2011) will further facilitate the screening and prioritization of blood-stage antigens for vaccine development in the future.

The extensive polymorphisms of proteins expressed on the infected erythrocyte (Guizetti *et al.* 2013) makes them challenging targets for vaccine development. However, there has been interest in the development of vaccines against pregnancy associated malaria (Hviid 2010) with particular focus on a variant of *P. falciparum* erythrocyte membrane protein 1 (*PfEMP1*) known as VAR2CSA (Rogerson *et al.* 2007) that mediates the sequestration of infected erythrocytes to the placenta. Parallel to these developments, are efforts to identify antibodies to different domains of VAR2CSA that may be broadly cross-reactive (Avril *et al.* 2010). Recently, it has been shown that severe malaria is associated with the expression of specific *PfEMP1* variants (Lavstsen *et al.* 2012). The same *PfEMP1* variants have also been shown to mediate the binding of parasitized erythrocytes to the brain endothelium *in vitro* (Avril *et al.* 2012; Claessens *et al.* 2012). These observations suggest that severe malaria may be mediated by a few *PfEMP1* variants that may be relatively conserved. In the future, further characterisation of these *PfEMP1* variants and identification of their endothelial receptors may reveal novel targets for vaccines against severe malaria.

### **1.7.3 Transmission blocking vaccines**

The presence of transmission blocking antibodies in individuals who are naturally exposed to *P. falciparum* (Graves *et al.* 1988; Bousema *et al.* 2006) together with the possibility of interrupting malaria transmission has sustained research interests in transmission blocking vaccines. Gametocyte proteins Pfs25, Pfs48/45 and Pfs230 are the targets of some of the leading vaccine candidates (Quakyi *et al.* 1987; Wu *et al.*

2008). Although, it is thought that transmission blocking vaccines will not, on their own, be efficacious in populations under high malaria transmission (Carter *et al.* 2000), these vaccines are attractive candidates for multi-component vaccines. One can postulate that combining pre-erythrocytic and/or erythrocytic vaccines with transmission blocking vaccines will be advantageous since the transmission blocking vaccines will prevent the transmission of any variants of the parasite that escape vaccine-induced pre-erythrocytic and erythrocytic immunity.

## **1.8 PLASMODIUM FALCIPARUM MEROZOITE ANTIGENS**

A panel of merozoite antigens, which are currently under development as vaccine candidate antigens and/or have observed immunogenicity, were selected and included in the studies presented in this thesis. These selected antigens are described in brief below.

### **1.8.1 Merozoite surface protein 1**

The merozoite surface protein 1 is an abundant protein on the surface of the merozoite first described by Holder and Freeman (Holder *et al.* 1981). It is synthesized as a 190 kDa precursor, which undergoes proteolytic cleavage such that by the time of erythrocyte invasion, only the C-terminal 19 kDa fragment (MSP-1<sub>19</sub>) remains on the merozoite surface (Blackman *et al.* 1990). At sequence level, MSP-1 is considered to have 17 blocks based on sequence variability (Tanabe *et al.* 1987).

Overall, MSP-1 sequences can be grouped into two major allele families represented by the KI and MAD20 parasite lines (Tanabe *et al.* 1987). Block 2 of MSP-1 has repeat sequence regions that make up 3 allelic families (Miller *et al.* 1993). MSP-1<sub>19</sub>, contained in block 17 and being the most conserved region of MSP-1, has at least 6 single nucleotide polymorphisms (SNPs).

### **1.8.2 Merozoite surface protein 2**

The merozoite surface protein 2 is a ~30 KDa glycoprotein anchored in the merozoites plasma membrane (Smythe *et al.* 1988). At the sequence level, the *msp2* gene has can be divided into 5 blocks (Snewin *et al.* 1991). The N- and C- terminal domains (blocks 1 and 5 respectively) are conserved whereas block 2 and 4 are made up of non-repetitive semi-conserved sequences. Diversity in the block 3 of *msp2*

consists of a repeat region that varies in length and sequence, flanked by dimorphic sequences that define two major allelic families, 3D7- and FC27-type (Smythe *et al.* 1991; Felger *et al.* 1994). Polymorphisms in the 3D7 family are due to repeat units that vary in sequence, length and copy number. Polymorphisms within the FC27 family are less diverse and consist of varying number of repeats of 36 and 96 bp, which results in fewer alleles in the FC27 family compared to the 3D7 family (Smythe *et al.* 1991; Felger *et al.* 1994). Point mutations have also been demonstrated in both allele families (Felger *et al.* 1994; Felger *et al.* 1997). The extensive diversity of block 3 of *msp2* is exemplified by the detection of over 50 genotypes by PCR-RFLP in each of the two allelic families 3D7 and FC27 (Felger *et al.* 1994; Felger *et al.* 1999).

### **1.8.3 Merozoite surface protein 3**

The merozoite surface protein 3 has a polymorphic N terminal sequence and a relatively conserved C-terminal sequence (McColl *et al.* 1997). Polymorphisms in the N terminal region are due to deletions and substitutions (McColl *et al.* 1997). Generally, *msp3* sequences can be grouped into two main allelic types 3D7- and K1-types (Huber *et al.* 1997).

### **1.8.4 Apical membrane antigen 1**

Apical membrane antigen 1 is a ~83 KDa protein made up of three domains that are stabilized by eight disulphide bonds (Hodder *et al.* 1996). The gene encoding AMA-1, unlike the *msp1*, *msp2* and *msp3* genes, has no repeats and polymorphisms are due to SNPs that are mostly located in domain 1. There is extensive polymorphism among sequences of genes coding for AMA-1. For instance, in one study 214 unique haplotypes of AMA1 were identified in 506 *P. falciparum* infections in a single geographical location in Mali (Takala *et al.* 2009). There is evidence that AMA-1 polymorphisms in malaria endemic areas are maintained by balancing selection that is probably mediated by host immunity (Polley *et al.* 2001; Cortes *et al.* 2003; Polley *et al.* 2003; Osier *et al.* 2010).

### **1.8.5 *Plasmodium falciparum* reticulocyte homologue 2**

The *Plasmodium falciparum* reticulocyte homologue 2 (PfRh2) protein is located in the rhoptries of the merozoite (reviewed in Cowman *et al.* 2012). PfRh2 has a region of ~500 amino acids that is identical to the *P. vivax* reticulocyte homologue 1 and 2

proteins (Rayner *et al.* 2000). The PfRh2a and PfRh2b genes are identical for their first 7.5 kb, but diverge greatly towards their C-termini, sharing only a putative membrane-spanning region and a cytoplasmic tail (Rayner *et al.* 2000). Polymorphisms in the *PfRh2* gene are predominantly located at the N-terminal region (Rayner *et al.* 2005; Reiling *et al.* 2010) and seem to be under balancing selection (Reiling *et al.* 2010).

## **1.9 ANTIBODY RESPONSES TO THE *PLASMODIUM FALCIPARUM* MEROZOITE SURFACE ANTIGENS**

The observation that high antibody titres to merozoite antigens are associated with protection from high-density parasitemia and clinical malaria but not from risk of reinfection (Stanisic *et al.* 2009; Richards *et al.* 2010) suggests that anti-merozoite antibodies may function by limiting parasitemia *in vivo*. Protection against clinical malaria has been mainly associated with IgG antibodies and appears to depend on the balance between the cytophilic (IgG1 and IgG3) and the non-cytophilic (IgG2 and IgG4) antibodies (Bouharoun-Tayoun *et al.* 1992). Anti-merozoite antibodies in individuals living in *P. falciparum*-endemic areas are predominantly of IgG1 and IgG3 isotypes (Taylor *et al.* 1995; John *et al.* 2005; Osier *et al.* 2007; Iriemenam *et al.* 2009; Stanisic *et al.* 2009; Richards *et al.* 2010; Awah *et al.* 2011; Nasr *et al.* 2011) which have also been associated with protection against clinical malaria (Aribot *et al.* 1996; Taylor *et al.* 1998; Richards *et al.* 2010). IgG2 responses to ring-infected erythrocyte surface antigen (RESA) and MSP-2 have been associated with reduced risk of *P. falciparum* infection (Aucan *et al.* 2000). Conversely, IgG4 antibodies have been associated with increased risk of clinical malaria and are thought to compete with cytophilic antibodies for receptors on monocytes (Aucan *et al.* 2000).

*P. falciparum*-specific antibodies mediate their function by binding to merozoite antigens to either directly inhibit erythrocyte invasion (Cohen *et al.* 1969; Brown *et al.* 1982; Hodder *et al.* 2001; McCallum *et al.* 2008) or, in cooperation with monocytes, lead to antibody dependent cellular inhibition (ADCI) of the intra-erythrocytic growth of the parasite (Khusmith *et al.* 1983). Antibodies in sera of individuals exposed to *P. falciparum* endemic areas have been shown to opsonize merozoites and to induce the release of reactive oxygen species from neutrophils in what has been referred to as antibody dependent respiratory burst (ADRB) (Joos *et al.*

2010). The observation that the magnitude of this ADRB activity correlates with protection against clinical malaria (Joos *et al.* 2010), coupled with enhanced neutrophil-dependent growth inhibition of *P. falciparum in vitro* by antibodies in immune sera (Kumaratilake *et al.* 1990) suggests that antibody-mediated activation of neutrophils is an effector function of anti-merozoite antibodies. Antibodies against asexual blood stages of the parasite can also opsonize merozoites and thus enhance their uptake by phagocytic cells (Groux *et al.* 1990) as well as neutralize pro-inflammatory molecules associated with merozoite antigens such as GPI (Naik *et al.* 2006).

The question as to whether antibody responses to specific merozoite antigens are protective against clinical malaria has been addressed by several immuno-epidemiological studies which have largely yielded inconsistent results (Fowkes *et al.* 2010). These inconsistencies may be attributable to differences in follow up time, antibody quantification methods and *P. falciparum* transmission intensities among prospective cohort studies (Fowkes *et al.* 2010). The difficulty in discriminating between individuals who are immune to clinical malaria from those who may be unexposed to the parasite also complicates the analysis of associations between anti-merozoite antibody responses and risk of malaria in these immuno-epidemiological studies (reviewed in Marsh *et al.* 2006). As a solution, Bejon *et al.* have proposed that individuals who remain aparasitemic be regarded as unexposed and be excluded from the analysis of associations between antibody responses and risk of malaria (Bejon *et al.* 2009). Several studies have subsequently demonstrated the value of this analytical approach (Kinyanjui *et al.* 2009; Bejon *et al.* 2011; Greenhouse *et al.* 2011). For instance, Greenhouse *et al.* have shown, by conditioning the risk of clinical malaria on being parasitemic at frequent time intervals, that antibody responses to the blood stage antigens AMA-1, MSP-1 and MSP-3 but not the pre-erythrocytic antigens CSP and liver-stage antigen 1 (LSA-1) are strongly associated with protection from clinical malaria (Greenhouse *et al.* 2011).

Additionally, some immuno-epidemiological studies investigating the association between anti-merozoite antibody responses and risk of malaria have classified individuals based on sero-positivity (a cutoff derived from antibody titres in sera from *P. falciparum*-naïve adults) (Fowkes *et al.* 2010) yet several studies have suggested that high anti-merozoite antibody titres are better predictors of protection against malaria than sero-positivity (John *et al.* 2005; John *et al.* 2008; Osier *et al.* 2008; Courtin *et*

*al.* 2009; Stanistic *et al.* 2009; Reiling *et al.* 2010; Richards *et al.* 2010; McCarra *et al.* 2011). A few studies have explored analytical approaches that account for antibody concentrations in the assessment of associations between anti-merozoite antibodies and risk of clinical malaria (John *et al.* 2005; John *et al.* 2008; Murungi *et al.* 2013). Future immuno-epidemiological studies employing these alternative analytical approaches may contribute towards rational screening and prioritization of blood-stage antigens for vaccine development.

## **1.10 GENETIC DIVERSITY OF *PLASMODIUM FALCIPARUM***

The development of a vaccine against *P. falciparum* is challenged, at least in part, by *P. falciparum*'s antigenic variation (reviewed in Scherf *et al.* 2008) and extensive genetic diversity in some of its antigens (reviewed in Takala *et al.* 2009). Further, the parasite continues to evolve through genetic mutations in response to host immunity (reviewed in Mackinnon *et al.* 2010). Due to the parasite's evolution in response to host immunity, it has been proposed that the deployment of malaria vaccines that reduce parasite replication *in vivo*, as opposed to those that prevent infection, may select for more virulent parasites and thus negate the population-wide benefits of such vaccines (Gandon *et al.* 2001; Mackinnon *et al.* 2008). Vaccine induced selection on parasite populations was suggested by the results of a randomized, placebo-controlled trial of the Combination B blood-stage vaccine that contained the 3D7-like allele of MSP-2, MSP-1 and ring-infected erythrocyte surface antigen (Genton *et al.* 2002). In that trial, subsequent infections among the vaccinees were predominantly due to parasites bearing the alternative FC27-like alleles of MSP-2 antigen (Genton *et al.* 2002). The design of vaccines that overcome the extensive genetic diversity of *P. falciparum* will require the understanding of vaccine antigen polymorphisms and their natural dynamics in malaria endemic areas.

### **1.9.1 Genetic diversity of asymptomatic *P. falciparum* infections**

Asymptomatic *P. falciparum* infections composed of multiple genetically-distinct clones are common in humans living in malaria endemic areas (Ntoumi *et al.* 1995; Felger *et al.* 1999; Smith *et al.* 1999; Berczky *et al.* 2007; Farnert *et al.* 2009). These infections are often seen in individuals who have been repeatedly infected and have gradually developed some degree of immunity against clinical malaria. *P. falciparum* infections are often characterized by extensive within-host dynamics such that different

parasite genotypes are detected in peripheral blood on a hourly (Farnert *et al.* 2008) or daily (Farnert *et al.* 1997) basis in otherwise healthy individuals.

The phenomenon of an almost permanent presence of low density parasitaemia in the presence of an immune response that is capable of preventing clinical symptoms was initially described by Koch and is often termed “premunity” (Sergent & Parrot 1935). The human host’s tolerance to infections by multiple genetically diverse clones of the parasite is one aspect that characterizes naturally acquired immunity to malaria (Doolan *et al.* 2009). Perignon and Druilhe have proposed that premunity is mediated by immune mechanisms characterized by the cooperation between monocytes and antibodies in what has been termed as antibody dependent cellular inhibition (ADCI) (Perignon *et al.* 1994). According to that proposition, ADCI is triggered by merozoites and acts on blood-stage parasites. Erythrocytic parasites mature uninhibited over several cycles until the number of merozoites reaches the threshold necessary to induce monocytes which in turn kill blood stage parasites and reduce parasitaemia. With blood stage parasites being both the trigger and target of ADCI, parasitaemia fluctuates at low densities as is observed in malaria endemic areas. The plausibility of that hypothesis in explaining the persistent presence of low density parasitaemia *in vivo* is supported, although indirectly, by the observation that ADCI inhibits parasite growth *in vitro* (Khusmith *et al.* 1983).

It has been proposed that the time required to achieve immunity to clinical malaria in *P. falciparum*-endemic areas (reviewed in Marsh *et al.* 2006), reflects the need to develop a progressively enlarging panel of antibody specificities that eventually enable recognition of several serologically diverse infections (Day *et al.* 1991). In concurrence with this proposition are several studies that have shown that clinical malaria in individuals in malaria endemic areas are associated with parasite genotypes not previously present in these individuals (Contamin *et al.* 1996; Kun *et al.* 2002). These observations suggest that clinical malaria results from exposure to new infections which are not recognized by host immune responses that are capable to controlling existing infections. A further inference would be that with more exposure comes the ability of the host immune responses to control a larger number of genetically distinct *P. falciparum* clones. The presence of multiple concurrently infecting clones can therefore be considered to reflect acquired immunity or premunity (Smith *et al.* 1999).

### **1.9.2 Genetic diversity of asymptomatic *P. falciparum* infections in relation to protection against clinical malaria**

The question as to whether the presence of multiple concurrently infecting clones reflects an individual's level of immunity has been addressed by several studies. Whereas some of the studies have shown that multiple concurrently infecting clones are associated with protection from disease (al-Yaman *et al.* 1997; Farnert *et al.* 1999; Muller *et al.* 2001; Berezky *et al.* 2004; Berezky *et al.* 2007; Liljander *et al.* 2010) several others have shown the opposite (Felger *et al.* 1999; Branch *et al.* 2001; Ofosu-Okyere *et al.* 2001; Mayor *et al.* 2003; Mayengue *et al.* 2009). These inconsistencies may be attributed to differences in different *P. falciparum* transmission intensities since the association between the multiclonality of *P. falciparum* infections and risk of malaria has been shown to be transmission-dependent (Farnert *et al.* 2009). The most apparent pattern is that, in areas of high *P. falciparum* transmission, asymptomatic multiclonal infections are associated with a reduced risk of subsequent clinical malaria (al-Yaman *et al.* 1997; Farnert *et al.* 1999; Berezky *et al.* 2007). On the other hand, in children younger than 3 years of age in high transmission areas or children of any age in low *P. falciparum* transmission settings, multiple concurrently infecting clones are associated with a higher risk of disease (Felger *et al.* 1999; Branch *et al.* 2001; Mayor *et al.* 2003; Mueller *et al.* 2012). Therefore, in older partially immune children in areas of high *P. falciparum* transmission the presence of multiclonal infections seems to be conferring protection against clinical malaria or be a marker of other protective mechanisms. In line with this observation, authors of a review on malaria immunity (Struik *et al.* 2004), have proposed that the inability of the individuals in malaria endemic areas to completely clear parasitaemia does not necessarily imply that naturally acquired immunity is defective. Conversely, they propose that naturally acquired immune mechanisms are adequate to prevent high density parasitaemia and clinical malaria yet allow for the persistence of the parasite at low-densities which may be useful for the maintenance of immunity. The importance of the persistent, rather than intermittent, presence of multiclonal infections for the maintenance of immunity to malaria was shown in a randomized placebo-controlled trial of intermittent preventive treatment in children in which multiclonal infections were associated with protection against malaria only in the placebo group (Liljander *et al.* 2010).

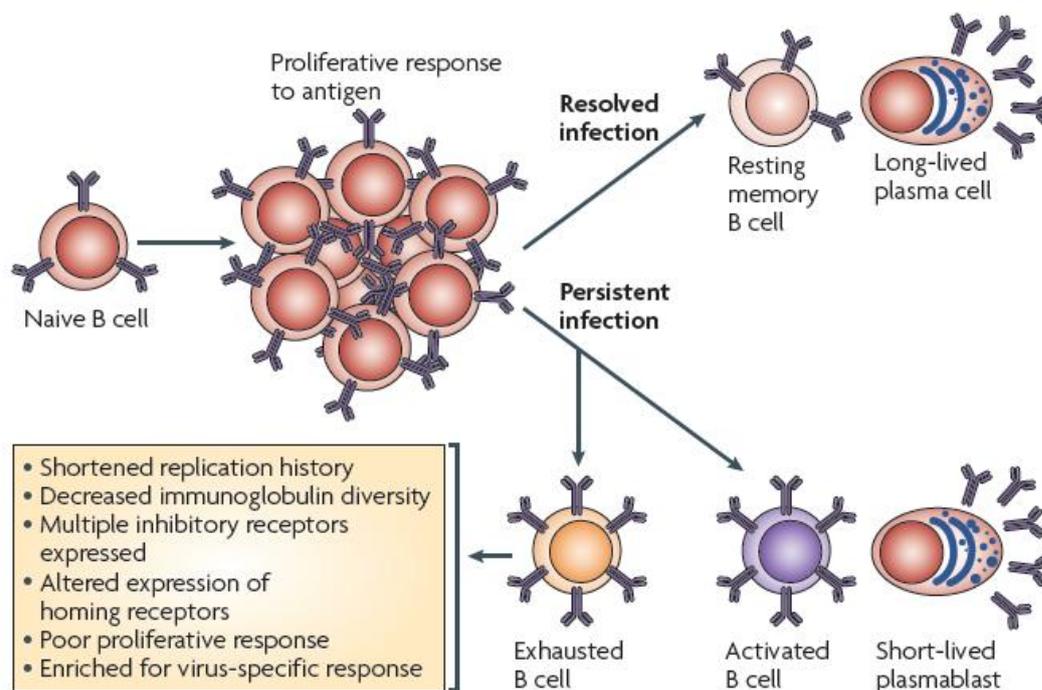
A practical implication of this interpretation is that malaria vaccines need not induce sterilizing immunity at least in areas of high *P. falciparum* transmission. This interpretation, however, is challenged by observations made by some studies that the presence of multiple concurrently infecting clones is associated with an increased risk of anemia in children (May *et al.* 2000; Mockenhaupt *et al.* 2003) and pregnant women (Beck *et al.* 2001) independently of parasite density.

The relationship between the genetic diversity of *P. falciparum* infections and other factors that are associated with immunity to clinical malaria such as antibody responses to merozoite antigens has been unclear and merits further investigation. On one hand, in a longitudinally monitored cohort on the coast of Kenya, the associations between high antibody titres to MSP-2 and reduced risk of clinical malaria were particularly strong in children whose antibodies were directed against the same dimorphic type of MSP-2 antigen as the concurrently infecting parasites (Polley *et al.* 2006) suggesting that protective antibody responses are allele-specific. On the other hand, in another study, although allele-specific antibodies to MSP-2 were present, there was no evidence that they were associated with a reduced risk of clinical malaria caused by parasites bearing the same MSP-2 allele (Osier *et al.* 2010).

### **1.11 IMMUNOLOGICAL MEMORY TO *PLASMODIUM FALCIPARUM* MEROZOITE ANTIGENS**

Antigen-specific immunological memory is a characteristic of adaptive immunity that mediates a faster and stronger immunological response upon re-exposure to antigen. Long-lived humoral immunity is dependent on the generation and maintenance of memory B-cells (MBCs) and long-lived plasma cells (LLPCs) (Gourley *et al.* 2004; Amanna *et al.* 2010) (Figure 4). Upon encounter with a foreign antigen, naïve marginal-zone B-cells residing in the margins of the B- and T-cell regions of secondary lymphoid organs (Pillai *et al.* 2005) differentiate into isotype switched plasmablasts (Lopes-Carvalho *et al.* 2004) which proliferate and secrete antibodies. In the context of infectious pathogens, these antibodies contribute to the initial resolution of infection. On the other hand follicular B-cells, upon encounter with antigen, migrate into germinal centers where they proliferate and undergo affinity maturation and class-switch recombination (McHeyzer-Williams *et al.* 2001). The germinal centre reaction lasts between 10 and 14 days after immunization and yields

MBCs and LLPCs with high-affinity B-cell receptors and switched antibody isotypes (Shapiro-Shelef *et al.* 2005). MBCs which by this time have acquired an inherent ability to respond and proliferate faster than naïve B-cells on secondary exposure to cognate antigen (Tangye *et al.* 2003), exit the germinal centers and circulate in blood where they mediate recall immune responses. LLPCs home to the bone marrow and constitutively secrete antibodies.



**Figure 4.** B-cell subsets in the context of acute and chronic infections. (Adopted from Moir *et al.*, 2009 and published with permission from Nature Publishing Group).

Antibody responses to several viral and vaccine antigens have been shown to be long-lived (with half-lives being longer than a decade) (Amanna *et al.* 2007). Conversely, *P. falciparum*-specific antibody responses seem to be relatively short-lived in children. Longitudinal studies, for instance, have shown that *P. falciparum*-specific antibodies rapidly decline below detection limits within a few months of an episode of clinical malaria (Fruh *et al.* 1991; Taylor *et al.* 1996; Cavanagh *et al.* 1998; John *et al.* 2002; Akpogheneta *et al.* 2008; Weiss *et al.* 2010). Further, the half-lives of IgG1 and IgG3 specific to merozoite antigens has been estimated to be 9.8 and 6.1 days respectively (Kinyanjui *et al.* 2007). These studies suggest that natural *P. falciparum* infections in children do not induce stable pools of LLPCs. Some studies have suggested that there is acquisition of more stable antibody responses with age (Riley

*et al.* 1993; Taylor *et al.* 1996; Wipasa *et al.* 2010). However, the interpretation of these studies is precluded by the inability to rule out the effect of on-going parasite exposure on the observed longevity of antibody responses.

A study done in an area of marked seasonal *P. falciparum* transmission showed that, whereas antibody titres increase and decrease during the high and low malaria transmission seasons respectively, the antibody titres at the end of the low transmission season remained higher than they were before the high malaria transmission season (Crompton *et al.* 2010). Antibody titres increased with each year of malaria transmission such that by 9 years of age antibody titres before and after the high transmission season were similar (Crompton *et al.* 2010). This observation suggests that the buildup of sufficient pools of *P. falciparum*-specific LLPCs necessary for the maintenance of stable antibody titres takes several years of *P. falciparum* exposure (Crompton *et al.* 2010).

The relative inefficiency in the acquisition of *P. falciparum*-specific LLPCs has been attributed to several mechanisms (reviewed in Portugal *et al.* 2013). Out of the proposed mechanisms, one of the best described relates to the dysregulation of B-cell differentiation. Several chronic infections have been associated with dysregulated B-cell differentiation (Moir *et al.* 2008; Moir *et al.* 2009; Sansonno *et al.* 2009). For instance, chronic HIV infection has been associated with the expansion of a pool of morphologically and functionally distinct memory B-cells (Moir *et al.* 2008). These memory B-cell subsets are characterized by the expression of an inhibitory receptor Fc-receptor-like-4 (FCRL4) and poor proliferation in response to polyclonal stimulation *in vitro* (Moir *et al.* 2008). Increased frequencies of phenotypically similar memory B-cells have also been associated with chronic *P. falciparum* infection (Weiss *et al.* 2009; Nogaro *et al.* 2011; Portugal *et al.* 2012; Illingworth *et al.* 2013; Muellenbeck *et al.* 2013). Whereas a causal link between *P. falciparum* exposure and expansion of atypical memory B-cell pools has not been established several observations point towards the existence of such a link. The proportion of these atypical memory B-cells as a fraction of total memory B-cells correlates with *P. falciparum* transmission intensity (Weiss *et al.* 2011) and contracts in the prolonged absence of *P. falciparum* infection (Ayieko *et al.* 2013). Further, comparison of the frequencies of memory B-cells between two populations of age-matched children that are otherwise similar except for differences in *P. falciparum* transmission intensity

showed a higher frequency in the population under high transmission (Illingworth *et al.* 2013). Additionally, a recent study has shown that the association between *P. falciparum* exposure and acquisition of atypical MBCs can be observed even at the single-cell level (Muellenbeck *et al.* 2013). The different IgG gene repertoires between the atypical and classical MBCs observed in that study suggested that the two cell types differentiate from different precursor cells (Muellenbeck *et al.* 2013). Interestingly, the atypical MBCs appeared to constitutively secrete antibodies that were broadly neutralizing to blood stage *P. falciparum* parasites (Muellenbeck *et al.* 2013). What remains to be established is whether antibodies constitutively expressed from atypical MBCs confer protection against clinical malaria.

## 2 AIM OF THE THESIS

The overall aim of this thesis was to contribute to the understanding of antibody responses to *P. falciparum* merozoite antigens in relation to the parasite's genetic diversity and natural acquisition of protective immunity. The thesis also aimed to study immunological memory to *P. falciparum* merozoite antigens.

### Specific aims:

The specific aims of the papers presented in this thesis were:

- I. To investigate the associations between the breadth of anti-merozoite antibody responses and the genetic diversity of asymptomatic *P. falciparum* infections in relation to protection against clinical malaria
- II. To investigate the relationships between the inhibitory activity of naturally acquired antibodies on the *in vitro* growth of *P. falciparum* and risk of clinical malaria and the dependence of this association on merozoite invasion phenotype.
- III. To describe the temporal dynamics of naturally acquired antibodies to a panel of *P. falciparum* merozoite antigens over a five-year period in children who experience different numbers of episodes of clinical malaria.
- IV. To investigate the longevity of *P. falciparum* merozoite antigen-specific antibodies and memory B-cells induced by natural infections.

## 3 MATERIALS AND METHODS

### 3.1 STUDY POPULATIONS

Three studies included in this thesis were conducted in populations living in *P. falciparum*-endemic areas on the coast of Tanzania (Study I and II) and Kenya (Study III). Study IV was done in travellers diagnosed with *P. falciparum* malaria in Sweden after returning from malaria-endemic countries

#### 3.1.1 Tanzania (Study I and II)

Study I and II were conducted within a longitudinally followed population in Nyamisati village, Rufiji District, Tanzania (Rooth 1992). The village experiences perennial malaria transmission with peaks coinciding with the two rainy seasons; April-June (long rains) and November-December (short rains). A population of about 1000 individuals in Nyamisati has been monitored between 1985 and 1999 by a research team that also operated the only health facility in the village. Study I and II were done on subsets of individuals participating in a cross-sectional survey conducted in March-April 1999 in which 890 individuals aged 1-84 years were recruited, sampled for whole blood and monitored for the subsequent 40 weeks for episodes of clinical malaria. At that cross-sectional survey, parasite prevalence in children aged 2-10 years (*PfPR*<sub>2-10</sub>) was 44% suggesting that the area was under high malaria transmission intensity. Study I was restricted to 320 children aged  $\leq 16$  years who were asymptomatic at the time of the survey. Study II is a case-control study that included 55 children who experienced at least one episode of malaria in the 40-weeks follow up period (cases) and 116 age-matched controls.

#### 3.1.2 Kenya (Study III)

This study was nested within a larger population in Ngerenya, located on the northern side of an Indian ocean creek on the coast of Kenya that has been previously described (Mwangi *et al.* 2005; Mwangi *et al.* 2008). The area experiences a long rainy season in May-July and a short rainy season in November. The study population has been longitudinally monitored since 1998 to date with active weekly surveillance of the entire population and both passive and active malaria case detection (Mwangi *et al.* 2005). Over this period, *P. falciparum* transmission has dramatically reduced from an

estimated 10 infective bites/person/year and parasite prevalence of 40% in 1998 (Mbogo *et al.* 1995) to a parasite prevalence of zero as from 2009 to date. This study was conducted in a subset of children who experienced 5-16 episodes of malaria between September 1998 and October 2003 who were age-matched to children who either did not experience malaria or had only 1 episode of malaria during the same period.

### **3.1.3 Travelers diagnosed with *Plasmodium falciparum* malaria in Sweden (Study IV)**

This study was conducted on individuals who had been admitted to Karolinska University Hospital with a *P. falciparum* malaria diagnosis between 1994 and 2010. Out of 270 invited patients, 47 consented to participate and visited the hospital for blood sampling. Out of the 47 patients, 33 were born in Europe whereas 12 were born in countries known to be endemic for *P. falciparum*. The median time since their malaria diagnosis and time of sampling was 11 (range 1-17) years. The study also included 8 Swedish adults with no prior exposure to *P. falciparum* and 14 adults with life-long residence in an area of high malaria transmission in Kenya.

## **3.2 ETHICAL CONSIDERATIONS**

Studies I and II were granted ethical approval by the National Institute for Medical Research in Tanzania. Study IV was ethically approved by the Kenya Medical Research Institute National Ethics Committee. All the studies were approved by the Regional Ethical Review Board in Stockholm, Sweden. Written informed consent was obtained from all adult participants and from the guardians of all participating children.

## **3.3 DETERMINATION OF THE GENETIC DIVERSITY OF ASYMPTOMATIC *PLASMODIUM FALCIPARUM* INFECTIONS**

The genetic diversity of asymptomatic *P. falciparum* infections was determined in studies I, II and III by genotyping the merozoite surface protein 2 gene (*msp2*) as previously described (Liljander *et al.* 2009). Briefly, the PCR included an initial amplification of the outer *msp2* domain, followed by two separate nested reactions with fluorescent primers targeting the two allelic types (families) of *msp2*: FC27 and IC (3D7), and fragment analysis by capillary electrophoresis in a DNA sequencer (3730, Applied Biosystems) and Gene Mapper software (Applied Biosystems). The number of

*msp2* genotypes identified by this method corresponds to the number of clones within an individual *P. falciparum* infection. In study I, *P. falciparum* infections that were found to contain only one *msp2* genotype were sequenced. The sequencing was done based on the *msp2* gene using BIGDYE Terminator version 3.1 (Applied Biosystems, UK) and an ABI 3130xl capillary sequencer (Applied Biosystems, UK). Sequences were assembled and aligned using Geneious version 6.1.6 (Biomatters, New Zealand).

### **3.4 QUANTIFICATION OF ANTI-PLASMODIUM FALCIPARUM ANTIBODIES**

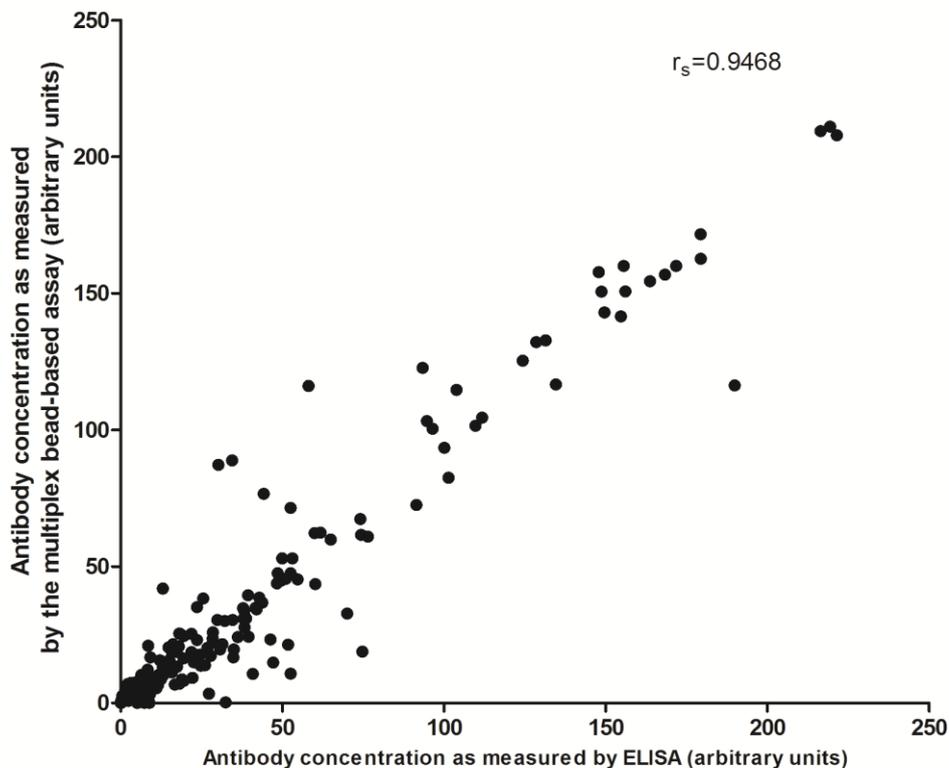
#### **3.4.1 Recombinant merozoite antigens**

Merozoite antigens used in all the studies included in this thesis were expressed as recombinant proteins in *Escherichia coli*. These antigens were selected because they are currently under development as vaccine candidate antigens and/or because of their observed immunogenicity (Taylor *et al.* 1996; Osier *et al.* 2008; Reiling *et al.* 2010). Apical membrane antigen 1 (AMA-1) from the 3D7 (Dutta *et al.* 2002) and FVO (Kocken *et al.* 2002) strains were expressed as 6xHis-fusion proteins. The 19-kilodalton fragment of merozoite surface protein (MSP) 1 (MSP-1<sub>19</sub>) (Burghaus *et al.* 1994), *P. falciparum* reticulocyte-binding homologue 2 (*PfRh2*) (Reiling *et al.* 2010), and two allelic forms of merozoite surface protein (MSP) 2; MSP-2\_Dd2 (corresponding to the FC27 allelic family of *msp2*) and MSP-2\_CH150/9 (corresponding to the IC-1 allelic family of *msp2*) (Taylor *et al.* 1995) were expressed as glutathione S-transferase-fusion proteins. MSP-3 antigens from the 3D7 and K1 strain were expressed as a maltose binding protein-fusion protein (Polley *et al.* 2007). All antigens, with the exception of *PfRh2* and MSP-1<sub>19</sub>, were expressed as full length proteins.

#### **3.4.2 Quantification of merozoite antigen-specific antibody responses using a multiplex bead-based assay**

A multiplex bead-based assay was adapted and optimized to simultaneously quantify plasma IgG antibodies to multiple *P. falciparum* antigens. To determine the optimal amount of antigen to use in the assay, one million Magplex beads (Luminex Corporation, USA) were coupled to 0.2, 1, 2.5 and 5µg of recombinant antigen using the Bio-plex amine coupling kit (Bio-Rad, USA). The antigen-coupled beads were then incubated with serial dilutions of a pool of hyperimmune sera (PHIS) taken from adults living in a malaria endemic area in Kenya. The results of this optimization step showed

that, for all the merozoite antigens tested, 1 $\mu$ g of recombinant antigen per a million beads was the least amount of antigen required to saturate all binding sites on the beads and that was uniform for all antigens. All subsequent optimization experiments were then done with this amount of antigen per a million beads. Further experiments with serial dilutions (1:50, 1:100, 1:150, 1:300 and 1:1000) of the secondary antibody (R-phycoerythrin-conjugated, F(ab')<sub>2</sub> fragment-specific, goat anti-human immunoglobulin G (Jackson ImmunoResearch Laboratories, USA) revealed that 1:300 dilutions were optimal. Comparison of the mean fluorescent intensities obtained when antigen-coupled beads were used in monoplex (single antigens) or multiplex (with up to 10 different antigens) showed that the assay gives comparable results in either format. Antibody titres in plasma samples from 100 Tanzanian children and adults to a panel of seven merozoite antigens were quantified using the multiplex bead based assay and the results compared to titres obtained using a standardized ELISA assay (Osier *et al.* 2008). A high level of correlation between the multiplex and ELISA data was observed as illustrated using data on antibody titres to the AMA-1\_FVO antigen (Figure 5).



**Figure 5.** Correlation between anti-AMA-1\_FVO antibody titres as measured by ELISA (x axis) and by the multiplex bead-based assay (y axis).  $r_s$  refers to Spearman's rank correlation coefficient.

In the final optimized assay, used in studies I and III, spectrally-unique beads were coupled at 5ng/5000 microspheres/well per antigen and plasma samples were assayed at 1:1000 dilution in Bio-Plex Pro flat bottom plates (Bio-Rad, USA). In study I, after incubation with secondary antibody, beads were analyzed on a Bio-Plex™ 200 (Bio-Rad, USA) controlled by a Bio-Plex Manager™ 6.0. software (Bio-Rad, USA). In study III, analysis was done using a Magpix instrument (Luminex, USA) and Xponent version 4.2. software (Luminex, USA). In both studies, the fluorescence intensity was read from at least 100 microspheres per spectral address/antigen and reported as mean fluorescence intensity (MFI). Serial dilutions of malaria immune globulin (MIG) (Taylor *et al.* 1992) were included in each plate as a standard positive control to correct for plate-to-plate variations and allow for the conversion of MFIs to relative antibody concentrations in arbitrary units.

### **3.4.3 Quantification of antibody responses to *Plasmodium falciparum* schizont extracts**

Antibody responses to *P. falciparum* schizont extracts were quantified using a previously described ELISA protocol (Ndungu *et al.* 2002). This protocol was modified by the addition of serial dilutions of malaria malaria-free globulin prepared by cold ethanol fractionation as previously described (Taylor *et al.* 1992) to each ELISA plate. This addition allowed for the conversion of optical densities to relative antibody concentrations in arbitrary units as well as for the correction of inter-plate variation.

## **3.5 QUANTIFICATION OF THE INHIBITORY ACTIVITY OF SERA ON THE *IN VITRO* GROWTH OF *PLASMODIUM FALCIPARUM***

In study II, inhibitory activity of sera on the *in vitro* growth of *P. falciparum* was quantified using a previously developed two-growth cycle assay (Persson *et al.* 2006). Briefly, *P. falciparum* parasites were cultured using human group O erythrocytes and synchronised using sorbitol before the start of the assay. Five µl of test serum was then mixed with 50 µl of parasite suspensions at 0.3% parasitemia and 1% hematocrit in 96-well U-bottom plates (Techno Plastic Products) and incubated for 2 parasite growth cycles (determined by the appearance of late ring and/or early trophozoite stages of the parasite in the second growth cycle). At the end of the 2 growth cycles, parasites were stained with dihydroethidium (Invitrogen Corporation, USA) and the number of infected erythrocytes quantified using a FACSCalibur cytometer (BD Bioscience, USA). CellQuest software (BD Bioscience, USA) was then used to determine the

percentage of infected erythrocytes. The GIA of each sera sample was then reported as percentage growth relative to parasite growth in control wells in which PBS or sera from *P. falciparum*-naive Swedish adults was added in place of the test sera sample.

Importantly, prior to their use in this assay, sera samples were dialyzed against PBS using 50-kDa molecular mass cut off (MMCO) dialysis tubes (G-Biosciences, USA) to exclude any inhibitory molecules or drugs that may interfere with the assay. Sera samples were then reconstituted to their original volumes using 100-kDa MMCO centrifugal concentration tubes (Pall Corporation, USA). The GIA was quantified against three parasite lines that have different erythrocyte invasion phenotypes as previously described (Triglia *et al.* 2005). The 3D7 line which was originally derived from NF54 invades erythrocytes by a predominantly sialic acid-independent invasion pathway that relies on PfRh2 and PfRh4. On the other hand, the W2mef line expresses more PfRh1 than 3D7 and uses a sialic acid-dependent pathway. The K1 line is an intermediate between 3D7 and W2mef in its dependence on sialic acid-dependent pathways for erythrocyte invasion.

### **3.6 PHENOTYPING MEMORY B-CELL SUBSETS**

Phenotypic analysis of memory B-cell subsets was performed by flow cytometry after surface staining with a panel of fluorophore-conjugated monoclonal antibodies as previously (Weiss *et al.* 2009). Monoclonal antibodies specific for the following markers were used: anti-CD10-allophycocyanin (APC), anti-CD20-APC-H7 (both from BD Biosciences, USA), anti-CD19-PerCP.Cy5.5 and anti-CD27-PE.Cy7 (both from eBioscience, USA). The Beckman Coulter CyAn ADP was used for flow cytometry and all data were analyzed using FlowJo software ((Tree Star, USA).

### **3.7 QUANTIFICATION OF *PLASMODIUM FALCIPARUM*-SPECIFIC MEMORY B-CELLS**

In study IV, *P. falciparum* merozoite antigen-specific memory B-cells were quantified using a modified and optimized ELISpot assay (Weiss *et al.* 2012) that was modelled on a previously developed assay (Crotty *et al.* 2004). Briefly, peripheral blood mononuclear cells are cultured for 5 days in the presence of polyclonal B-cell stimuli (a cocktail of CpG oligonucleotide (Eurofins MWG/Operon, USA), Protein A from *Staphylococcus aureus* Cowan (SAC), pokeweed mitogen (Sigma-Aldrich, USA) and

interleukin 10 (R&D Systems, USA). PBMCs from the 5-day cultures were then serially diluted and transferred to ELISpot plates pre-coated with; polyclonal goat antibody specific for human IgG (to detect total IgG secreting cells), bovine serum albumin (used as a negative control to correct for non-specific protein binding), tetanus toxoid (used as positive control), MSP-1, MSP-3 or AMA1. After a 5 hour incubation at 37°C in 5% CO<sub>2</sub> plates were washed and incubated further with 1:1000 dilution of alkaline phosphatase-conjugated goat antihuman IgG Fc antibodies. Plates were then developed using 100ul/well of BCIP/NBT and spots quantified using an ImmunoSpot series 4 analyzer (Cellular Technologies LTD, Germany) and results analyzed using Immunospot version 5 software (CTL, Germany). The results were reported as frequencies of MBCs per 10<sup>6</sup> PBMCs.

### 3.8 STATISTICAL ANALYSES

Data analysis in studies I – IV was done using STATA version 11.2. and Prism GraphPad version 5.02. R version 2.7.2. was used in Study I.

In studies I – IV, Proportions were compared using Chi-squared tests. Continuous variables between groups were compared using the Kruskal-Wallis or Mann-Whitney tests where appropriate.

In study I the relationships between the number of *P. falciparum* clones and breadth of anti-merozoite antibody responses and risk of malaria were estimated using modified Poisson regression models (Zou 2004) adjusting for the potential confounding effects of age and antibody responses to *P. falciparum* schizont extract (a proxy for exposure). The pARtial package (Lehnert-Batar 2006) within R was used to estimate attributable fractions and their associated CIs.

In study II Spearman's rank correlation coefficients were used to assess the relationships between continuous variables. Comparisons of the distribution of GIA and anti-schizont extract antibody concentrations across parasite lines were tested using the Friedman and Wilcoxon signed-rank tests. In light of the case-control design of the study, associations between GIA and the risk of malaria were assessed using conditional logistic regression.

In study III differences in rates of change in antibody titres over a 5-year study period were tested using a multilevel mixed-effects linear regression model that accounts for inherent correlations between repeated measurements done on the same subject (Rabe-Hesketh 2005).

In study IV, the predictive value of origin of the travellers, time since malaria diagnosis, age and parasitemia at time of malaria admission on the level of *P. falciparum*-specific antibody and memory B-cells were estimated using multivariable linear regression models.

## 4 RESULTS

### 4.1 STUDY I

#### **Breadth of anti-merozoite antibody responses is associated with the genetic diversity of asymptomatic *Plasmodium falciparum* infections and protection against clinical malaria**

Among the 890 individuals participating in the March-April 1999 cross-sectional survey, 320 children aged 1-16 years who were asymptomatic (without fever or other symptoms of malaria at the time of the survey, four weeks preceding or one week after the survey) were included in this study. We found that the number of genetically-distinct *P. falciparum* clones, determined by genotyping and sequencing the *msp2* gene, increased with age up to 5–10 years and stabilized thereafter. Similarly, the breadth of anti-merozoite antibody responses (defined as the number of antigens to which an individual had high antibody titres) increased with age (Kruskal-Wallis test  $P < 0.001$ ). The overall protection against malaria attributable to multiclonal infections and the breadth of anti-merozoite antibody responses decreased and increased with age respectively.

An increasing number of genetically-distinct *P. falciparum* clones was associated with reduced risk of malaria in univariate analysis and a similar trend was evident after age adjustment. Similarly, increase in the breadth of anti-merozoite antibody responses (defined as the number of antigens to which an individual had high antibody titres) was associated with a significant reduction in the risk of malaria independent of age and antibody reactivity to *P. falciparum* schizont extract. The number of clones in asymptomatic infections correlated positively with the breadth of anti-merozoite antibody responses (RR 1.63, 95% CI, 1.32-2.02). In combination, broad anti-merozoite antibody responses and genetically-diverse infections predicted a higher level of protection against clinical malaria than they did individually (RR 0.14, 95% CI, 0.04-0.48).

## 4.2 STUDY II

### ***Plasmodium falciparum* line-dependent association between growth-inhibitory activity and risk of malaria**

In a case-control study nested within a longitudinally monitored population in Tanzania, plasma samples collected at baseline from 55 cases and 116 randomly selected age-matched controls were assayed for growth-inhibitory activity (GIA) using three *P. falciparum* lines (3D7, K1, and W2mef) that have different erythrocyte invasion phenotypes. Antibodies to *P. falciparum* schizont extracts prepared from the cultures of these parasite lines were quantified by ELISA. We found no significant correlation between GIA to any of the parasite lines and age among the cases and only weak correlations among the controls. In contrast to GIA data, antibody titres to parasite schizont extracts of all the parasite lines increased significantly with age (Spearman's rank test  $r_s$ ,  $P < 0.001$ ). The correlation between GIAs determined using the three parasite lines was weak and non-significant among the controls and cases respectively. The distributions of GIA were significantly different among the three parasite lines with GIA against 3D7 being significantly higher than GIA against K1 and W2mef parasite lines (Wilcoxon signed-rank test  $P < 0.001$ ). The distributions of antibody titres to parasite schizont extracts of the three parasite lines were, however, similar (Friedman test  $P = 0.86$ ). Interestingly, GIA determined using the 3D7 parasite line was associated with a reduced risk of malaria OR 0.45 (95%, CI 0.21 - 0.96) whereas using the K1 and W2mef parasite lines, there was no association between GIA and the odds of experiencing malaria during follow-up. There was no significant difference in the breadth of GIA (i.e. the number of parasite lines to which an individual had GIA above the median) between the cases and controls (Chi-square test for trend  $P = 0.92$ ).

### 4.3 STUDY III

#### **Five-year temporal dynamics of naturally acquired antibodies to *Plasmodium falciparum* merozoite antigens in children experiencing multiple episodes of clinical malaria**

This study was nested within a larger longitudinally monitored cohort in an area of low malaria transmission on the coast of Kenya in which the distribution of the incidence of clinical malaria has been shown to be over-dispersed (Mwangi *et al.* 2008). A subset of children who experienced 5-16 episodes of malaria between September 1998 and October 2003 (i.e. children at the tail end of this over-dispersed distribution and herein referred to as the susceptible group) were identified and matched to children who remained free from malaria (malaria-free group) and children who experienced only one episode of malaria over the same 5-year period (intermediate group). Antibody responses to 7 merozoite vaccine candidate antigens were measured in plasma samples obtained from these children at 6 cross-sectional surveys that spanned the 5-year study period. The genetic-diversity of asymptomatic *P. falciparum* infections was assessed by genotyping the *mSP2* gene in samples of packed cells obtained at the same surveys. Temporal dynamics in antibody responses were described and quantified to identify variations in antibody responses that were associated with the different malaria susceptibility profiles of the 3 groups of children. We found that antibody responses to merozoite antigens were generally similar in children who experienced multiple malaria episodes compared to children who either remained free from malaria or experienced only a single episode. Overall, children in all three groups maintained relatively low antibody titres to the antigens tested with only transient peaks coinciding with asymptomatic infections. There was some indication of allele-specific boosting of antibody responses to MSP-2 whereby cross-sectional surveys at which children were infected with IC-1 or FC types of *mSP2* genotypes, were also associated with transient peaks of antibody titres to the corresponding MSP-2 type antigen. With the exception of AMA-1\_FVO, in which the rate of buildup of antibody responses with time was faster in the susceptible relative to the intermediate group ( $\beta$  coefficient -6.32, 95% CI -12.64 – -0.01), there were no significant differences in the temporal rates of change of antibody responses to other merozoite antigens in the 3 groups of children despite higher parasite exposure in the susceptible group compared to the intermediate and malaria-free groups. Children in the susceptible group had more genetically-diverse infections compared to children in the intermediate and malaria-free groups.

#### 4.4 STUDY IV

##### **Long-lived *Plasmodium falciparum*-specific memory B cells in naturally exposed Swedish travelers**

Levels of *P. falciparum* merozoite antigen-specific antibodies and memory B-cells as well as relative proportions of memory B-cell (MBC) subsets between 47 travelers who had been admitted with *P. falciparum* malaria at the Karolinska University Hospital between 1 and 16 years previously, 8 *P. falciparum* -naive adult Swedes (malaria-naïve adults), and 14 adults living in an area of high malaria transmission in Kenya (malaria-immune adults) were compared. *P. falciparum*-specific antibodies and MBCs were measured against a 1:1 mixture of two alleles of AMA-1 (AMA-1\_FVO and AMA-1\_3D7), the 42KDa C-terminal fragment of merozoite surface protein 1 (MSP-1<sub>42</sub>), and MSP-3. Antibody titres to *P. falciparum* parasite lysate were also quantified. We found that apart from the *P. falciparum* lysate-specific antibody titres where 30% of the travelers had levels above naive controls, antibody responses to all the merozoite antigens tested were at background levels among this group. On the contrary, 59, 45, and 28% of travelers had MBCs specific for AMA-1, MSP-1<sub>42</sub>, and MSP3 respectively. Further, 78% of the travellers had MBCs specific for at least one merozoite antigen. Interestingly, 5 travellers who had not left Sweden since their first malaria diagnosis had maintained MBCs specific for at least one merozoite antigen for a median of 12 (range 8 -16) years thus providing evidence for long-term maintenance of MBCs in the complete absence of re-exposure to the parasite. None of the *P. falciparum*-specific MBC responses were associated with time elapsed since malaria diagnosis, parasitaemia at diagnosis, previous malaria episodes or being born in a malaria-endemic country. Malaria-immune adults had an expanded atypical MBC compartment relative to the travellers and malaria-naïve adults. There was no difference in the relative proportions of atypical MBC between the travellers and malaria-naïve adults. The relative proportions of activated B-cells, classical MBCs, plasma cells, immature and naïve B-cells were similar in the three study groups.

## 5 DISCUSSION

Despite the decline in the global estimates of mortality attributable to *P. falciparum* malaria over the past decade (Murray *et al.* 2012), the disease still claims the lives of an estimated 660,000 people each year (WHO 2012) and remains a significant public health concern particularly in sub-Saharan Africa. The development of an effective anti-malarial vaccine is widely regarded as a major global health priority (reviewed in Crompton *et al.* 2010) but, this far, there is no licensed malaria vaccine. The acquisition of immunity to clinical malaria following repeated natural exposure to *P. falciparum* is a strong justification for the development of malaria vaccines that aim to mimic naturally acquired immunity (Crompton *et al.* 2010; Richards *et al.* 2013). The development of a malaria vaccine on this premise is hampered, at least partly, by the incomplete understanding of the immune mechanisms that mediate naturally acquired immunity. For instance, whereas passive antibody transfer studies have demonstrated the importance of an antibody-mediated component of naturally acquired immunity to malaria (Cohen *et al.* 1961; Sabchareon *et al.* 1991), the specific targets and effector mechanisms of most antibodies, such as those to merozoite antigens, are largely unknown (Fowkes *et al.* 2010).

The studies presented in this thesis, contribute to the understanding of naturally acquired immunity to malaria. In particular, they investigated different aspects of antibody responses to merozoite antigens and the genetic diversity of *P. falciparum* infections in the asymptotically infected human host in relation to risk of clinical malaria. The importance of antibody function to protection against malaria was explored in relation to erythrocyte invasion phenotypic differences between *P. falciparum* lines. Further, a comparison of the temporal dynamics of naturally acquired antibody responses between children who experience multiple malaria episodes and those who do not was assessed to further understand immune responses associated with increased susceptibility to disease. Also presented here are data on the longevity of *P. falciparum*-specific antibody and memory B-cell responses induced by natural infections.

Premunition, defined as immunity against clinical symptoms while chronically infected, has been described as a common phenomenon in humans living in malaria endemic areas (Sergent *et al.* 1935; Smith *et al.* 1999). Based on this observation, it has

been postulated (but not empirically tested) that the tolerance of multiclonal *P. falciparum* infections is associated with a broad repertoire of immune responses which control parasitemia and prevent malaria (Smith *et al.* 1999). Study I, in testing this hypothesis, shows that in an area of high malaria transmission, children who have the highest degree of protection against malaria have antibody responses to an increasing number of merozoite antigens and harbour an increasing number of genetically-diverse asymptomatic *P. falciparum* infections. Further, our data show that the multiclinality of asymptomatic infections correlates positively with the breadth of anti-merozoite antibody responses. These findings suggest that naturally acquired immunity is characterized by the presence of broad antibody responses (demonstrated here by broad anti-merozoite antibody responses) and the maintenance of low-densities of genetically different parasites rather than immune-driven elimination of the parasite. These results suggest that low levels of antigen may be required to maintain long-lasting antibody responses. Further, the observed increase in protection with increasing breadth of antibody responses supports the development of vaccines consisting of multiple antigens. There have been concerns about the feasibility of developing a vaccine that can overcome the extensive antigenic diversity that characterizes several of *P. falciparum* vaccine targets (reviewed in Crompton *et al.* 2010). Nonetheless, several studies have suggested that antigenic diversity can be overcome using only a few carefully selected alleles of the polymorphic antigens in a multicomponent vaccine (Mamillapalli *et al.* 2006; Remarque *et al.* 2008; Drew *et al.* 2012; Miura *et al.* 2013).

The importance of asymptomatic infections in relation to antibody-mediated immunity to malaria has been unclear. Whereas, some studies have suggested that asymptomatic parasitaemia does not influence associations between antibody responses and risk of malaria (Stanisic *et al.* 2009; Richards *et al.* 2010), others have shown that anti-merozoite antibodies are associated with protection from malaria only in parasitemic children (Polley *et al.* 2004; Polley *et al.* 2006; Osier *et al.* 2007; Osier *et al.* 2008; Greenhouse *et al.* 2011). A recent study has shown that the lack of a protective association in the aparasitemic children may be due to antibodies in this subset of children being lower than threshold antibody concentrations that seem to be necessary to confer protection against clinical malaria (Murungi *et al.* 2013) thus suggesting that concurrent parasitemia is important for the maintenance of anti-merozoite antibodies at high enough levels to confer protection against malaria. Our finding of a positive correlation between the multiclinality of asymptomatic infections and breadth of anti-

merozoite responses adds to these observations by suggesting that, not only are asymptomatic infections important, but their genetic diversity is important in relation to the breadth of antibody responses.

The limited knowledge of the specific targets and effector mechanisms that mediate immunity to malaria is partly attributable to the lack of functional immunological assays that clearly correlate with protective immunity. For instance, the associations between protection against clinical malaria and antibody function as measured by the, the growth inhibition assay (GIA), which is the most widely used functional antibody assay (Brown *et al.* 1982; Duncan *et al.* 2012), have been inconsistent. Whilst, some studies have reported significant associations between GIA and reduced risk of malaria (John *et al.* 2004; Dent *et al.* 2008; Crompton *et al.* 2010) other studies have been inconclusive (Marsh *et al.* 1989; Corran *et al.* 2004; Perraut *et al.* 2005; McCallum *et al.* 2008; Murhandarwati *et al.* 2009). Study II shows that the distribution of GIA and its association with protection against clinical malaria is dependent on *P. falciparum* parasite line. These findings imply that the choice of parasite line is important in vaccine and epidemiological studies in which GIA is used as a measure of vaccine efficacy or correlate of protection. Further, considering the biological complexity of *P. falciparum* infections, it is likely that protective immunity against clinical malaria is mediated by multiple effector mechanisms that are not adequately measured by existing functional assays. In the future, application of systems immunology (recent advances in biomedical research that aim to integrate data generated from high throughput molecular and genomic and cellular assays to identify biological factors associated with a phenotype or outcome of interest (Benoist *et al.* 2006; Tran *et al.* 2012)) to meticulously monitored populations may be necessary to identify immunological signatures that are predictive of immunity against malaria.

Heterogeneity in the risk of clinical *P. falciparum* malaria within human populations in malaria-endemic areas is widely described (Greenwood *et al.* 1987; Snow *et al.* 1988; Greenwood 1989; Trape *et al.* 2002; Brooker *et al.* 2004; Creasey *et al.* 2004; Ernst *et al.* 2006; Gaudart *et al.* 2006; Clark *et al.* 2008; Kreuels *et al.* 2008; Mwangi *et al.* 2008; Yeshiwondim *et al.* 2009; Bejon *et al.* 2010; Bousema *et al.* 2010). Foci of high malaria incidence have been proposed as attractive opportunities for targeted malaria control measures (Bousema *et al.* 2010; Bousema *et al.* 2012). However, remarkably little is known as to how individuals who experience multiple episodes of malaria

compare with those who either remain free from malaria or experience only few episodes in terms of their ability to mount immune responses following natural *P. falciparum* exposure or vaccination. Knowledge on this is important because the success of targeted deployment of vaccines to these individuals is dependent on their ability to respond optimally to antigen challenge. In study III, we observed that a subset of children who experienced multiple clinical episodes of malaria (here referred to as “susceptible” children) did not appear to differ from those who either remain free from malaria or experience fewer episodes of malaria in their ability to acquire immunity, at least as assessed by antibody responses to *P. falciparum* merozoite antigens. This observation is promising because children who experience multiple clinical malaria episodes appear to respond to natural *P. falciparum* infection (and are therefore expected to respond to vaccination) just as well as children in the general population. Whether these children are more susceptible to malaria due to deficiencies in other protective immune mechanisms is still unknown. Future studies based on systems immunology (Benoist *et al.* 2006; Tran *et al.* 2012) may be useful in identifying immunological signatures, if any, that are unique to this susceptible group of children.

The development of immunity to clinical malaria following repeated natural exposure to *P. falciparum* is a strong justification for the development of malaria vaccines that mimic naturally acquired immunity (reviewed in Crompton *et al.* 2010). This justification is challenged by previous studies that have suggested that natural *P. falciparum* infections, especially in children, may not induce long-lived immune responses (reviewed in Struik *et al.* 2004; Langhorne *et al.* 2008). However, data on the precise longevity of memory B-cell response to *P. falciparum* antigens has, so far, been limited partly because studies of the longevity of *P. falciparum* specific MBCs in malaria-endemic areas are precluded by ongoing *P. falciparum* transmission and the seasonal nature of malaria transmission in some malaria-endemic areas. For instance, whereas Weiss *et al.* (Weiss *et al.* 2010) showed that MBCs, acquired during 6 months of high malaria transmission, contracted over the subsequent 6 months of reduced malaria transmission, they could not study longevity beyond the dry season, as it was interrupted by the next high transmission season. Nonetheless, several studies that have quantified MBCs in areas of very low malaria transmission (Wipasa *et al.* 2010; Ndungu *et al.* 2012) or following a reduction in malaria transmission by indoor residual spraying (Ayieko *et al.* 2013) suggest that MBCs are maintained in the absence of infection. In study IV, we observed that in 78% of the travelers *P. falciparum*-specific MBCs to at

least one merozoite antigen were maintained for between 1 and 17 years. This observation provides the strongest evidence so far that natural *P. falciparum* infections can induce long-lived MBCs. The observation that some travelers in this study, as well as some individuals in previous studies (Weiss *et al.* 2010; Wipasa *et al.* 2010; Nogaro *et al.* 2011; Ndungu *et al.* 2012) were negative for *P. falciparum*-specific MBCs in spite of exposure to the parasite is intriguing and merits investigation in future studies. Additionally, future studies should take advantage of high throughput methods of screening of MBC repertoires, such as that developed by Traggiai *et al.* (Traggiai *et al.* 2004), to identify MBC specificities that are associated with protection against clinical malaria. These protective MBC specificities may be promising targets of future vaccines.

## 6 CONCLUSIONS AND FUTURE PERSPECTIVES

- Asymptomatic *P. falciparum* infections with an increasing number of genetically distinct clones are associated with an increasing breadth of antibody responses to merozoite antigens.
- In combination, multiclonal *P. falciparum* infections and the breadth of anti-merozoite antibody responses are associated with a lower risk of clinical malaria than they are individually.
- Growth-inhibitory activities of plasma from individuals living in an area of high malaria transmission, as well as its association with prospective risk of malaria are dependent on *P. falciparum* line and can be explained by erythrocyte invasion phenotypic differences between parasite lines.
- Children who experience multiple episodes of clinical malaria do not appear to differ from children who either remain free from malaria or experience fewer episodes of malaria in their ability to acquire and maintain antibody responses to *P. falciparum* suggesting that other factors such as differences in the intensity of exposure to the parasite may explain the differences in disease susceptibility.
- Natural *P. falciparum* infections can induce *P. falciparum*-specific MBCs that can be maintained for up to 16 years (or more) independently of sustained exposure to the parasite.

The development of vaccines against *P. falciparum* will benefit from a better understanding of the immune mechanisms that mediate naturally acquired immunity to malaria. Although the understanding of naturally acquired malaria immunity has been advanced by several studies over the past years, there are still several aspects of it that remain poorly understood. Taken together, the studies presented in this thesis have provided insights into naturally acquired antibody responses against the parasite as well as immunological memory induced by natural infections. In the future, application of systems immunology approaches to meticulously monitored populations in malaria-endemic areas may be necessary to further understand immunity to malaria. On one hand, application of these approaches to the parasite's proteome will facilitate the

screening and prioritization of *P. falciparum* antigens for vaccine development. On the other hand, these approaches will contribute to the identification of signatures of the human immune response that may be predictive of immunity against malaria. Further, future studies should take advantage of high throughput methods of screening MBC repertoires to identify MBC specificities that may be associated with protection against clinical malaria. These protective MBC specificities may be promising targets of future vaccines.

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