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**IMMUNITY TO MALARIA:
IMPORTANCE OF EXPOSURE AND PARASITE
DIVERSITY**

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IMMUNITY TO MALARIA:
Importance of Exposure and Parasite Diversity

THESIS FOR DOCTORAL DEGREE (Ph.D.)

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ABSTRACT

Malaria continues to carry an intolerable burden of disease and mortality, predominantly on children in Sub-Saharan Africa. An efficacious vaccine would be a powerful tool in the combat against malaria. Further understanding of the mechanisms of naturally acquired immunity to the polymorphic *Plasmodium falciparum* parasite would aid vaccine development.

The studies presented in this thesis contribute to the understanding of how immune responses are acquired in relation to exposure to the polymorphic *P. falciparum* parasite. More specifically, they investigated how the genetic diversity of the *P. falciparum* parasites encountered during childhood affects the risk of malaria (uncomplicated and severe), as well as whether maintenance of immune memory against *P. falciparum* antigens is maintained without repeated re-exposure. The thesis also included a description of a novel technique for dispersion of frozen blood clots for optimized DNA extraction and polymerase chain reaction (PCR).

Immunity against severe malaria has been proposed to be acquired after single infections. A matched case-control study was nested within a birth cohort on the Kenyan Coast where three-monthly blood samples up to two years, with the aim to study how exposure patterns early in life affects the risk of developing severe malaria. *P. falciparum* infections detected at least once from birth conferred an increased risk of severe malaria and especially if infections with two or more parasite clones were ever detected.

To optimize DNA extraction for the study above, a novel method was developed for dispersion of frozen residual clots saved from blood collected without anticoagulant for serum preparation. Compared to two commercial methods, high-speed shaking was most successful in clot dispersion before DNA extraction and generated the highest sensitivity of PCR detection.

Whether the genetic diversity of asymptomatic *P. falciparum* infections carried through the dry season affects the risk of malaria in the ensuing transmission season was investigated in a setting with intense but strictly seasonal malaria transmission in Mali. Individuals with multiclonal infections before the malaria season had a delayed time to their first febrile malaria episode and a lower incidence of malaria compared to individuals who were smear negative or only carried one parasite clone.

The longevity of immune responses to malaria after a single exposure was assessed in travelers treated for malaria in Stockholm, Sweden, 1-16 years previously. *P. falciparum*-specific memory B-cells were maintained in a majority of study participants for up to 16 years without re-exposure, whereas circulating cognate antibodies were not detected.

In conclusion, a single encounter with the parasite can induce immunological memory to *P. falciparum* antigens that lasts for many years. Severe malaria can occur in young children despite previous exposure, a finding that argues against the idea that severe malaria develops only after the first encounters with the parasite. Persistent asymptomatic infections, especially with genetically diverse infections, contribute to maintenance of immune responses that protect from malaria. This thesis presents different concepts and mechanisms of naturally acquired immunity to malaria that might be valuable in the development of malaria vaccines and other control strategies.

LIST OF PUBLICATIONS

- I. **Klara Lundblom**, Linda Murungi, Victoria Nyaga, Daniel Olsson, Josea Rono, Faith Osier, Edna Ogada, Scott Montgomery, Anthony Scott, Kevin Marsh and Anna Färnert
Plasmodium falciparum infection patterns since birth and risk of severe malaria: a nested case-control study in children on the coast of Kenya
PLoS One. 2013 Feb 8;2

- II. **Klara Lundblom**, Alex Macharia, Marianne Lebbad, Adan Mohammed and Anna Färnert
High-speed shaking of frozen blood clots for extraction of human and malaria parasite DNA.
Malaria Journal. 2011 Aug 8;10:229

- III. **Klara Sondén**, Safiatou Doumbo, Ulf Hammar, Manijeh Vafa-Homann, Assaita Ongoiba, Boubacar Traoré, Matteo Bottai, Peter D. Crompton and Anna Färnert
Asymptomatic multiclonal *Plasmodium falciparum* infections carried through the dry season predict protection against clinical malaria in the following high transmission season
In manuscript

- IV. Francis 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.
European Journal of Immunology. 2013 Nov;43(11):2919-29

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LIST OF ABBREVIATIONS

ACT	Artemisinin-based combination therapy
AMA	Apical membrane antigen
Bp	Base pair
CI	Confidence interval
CM	Cerebral malaria
DMSO	Dimethyl sulphoxide
EDTA	Ethylenediaminetetraacetic acid
EIR	Entomological inoculation rate
ELISA	Enzyme-linked immunosorbent assay
FACS	Fluorescence-activate cell sorting
IQR	Inter quartile range
Hb	Hemoglobin
HR	Hazard ratio
Ig	Immunoglobulin
IPT	Intermittent preventive treatment
LLIN	Long-lasting insecticide treated net
LLPCs	Long-lasting plasma cells
MBCs	Memory B-cells
MOI	Multiplicity of infection
molFOI	Molecular force of infection
MSP	Merozoite surface protein
OR	Odds ratio
PBMC	Peripheral blood mononuclear cells
PCR	Polymerase chain reaction
RBC	Red blood cell
RD	Respiratory distress
RDT	Rapid diagnostic test
RPMI	Roswell Park Memorial Institute medium
SMA	Severe malaria anemia
WHO	World Health Organization

1 INTRODUCTION

1.1 THE BURDEN OF MALARIA

Malaria remains a major health threat to humans in more than 90 countries (Figure 1). Despite scale-up of interventions between 2000 and 2012 there were an estimated 207 million clinical cases of malaria and 627 000 deaths, of which 483 000 in children, during 2012, according to the World Health Organization (WHO 2012). Children and pregnant women are at greatest risk and most deaths occur in Eastern, Central and Western sub-Saharan Africa (WHO 2013).

However, malaria-attributable mortality may be substantially under-estimated.

When Murray and colleagues used additional data from verbal autopsies malaria-attributable deaths were estimated to 1,113,000 (range 848,000-1,591,000) during 2010 (Murray *et al* 2012), whereas the same number estimated by WHO was 660,000 (range 490,000-836,000) (WHO 2012).

Nevertheless, both reports agree that there has been a global decline in malaria morbidity and mortality over the last decade, in parallel with the roll out of massive anti-malarial interventions such as artemisinin-based combination therapy (ACT) for the treatment of malaria cases, rapid diagnostic tests (RDT), intermittent preventive treatment (IPT) for selected groups and long-lasting insecticide treated bed nets (LLIN) (reviewed by Alonso *et al* 2013, Flaxman *et al* 2013).

The decrease in malaria morbidity and mortality has however not been uniform. Reports from Uganda and Malawi indicate that malaria hospital admission rates have increased during 1999-2009 and 2000-2010, respectively (Okiro *et al* 2011, 2013). In Kenya changes in pediatric malaria admissions were heterogenous with

a dramatic decline in the Kilifi District on the Kenyan coast and a significant increase of malaria admissions in other parts of the country (Okiro et al 2009).

The burden of malaria extends beyond health issues. Many malaria endemic countries are among the poorest in the world and malaria will impede development by high costs both for governments and individuals. Conversely, poverty itself contributes to some of the intense malaria transmission that exists in many of the poorest countries as well as an increased risk on an individual level (Sachs and Malaney 2002).

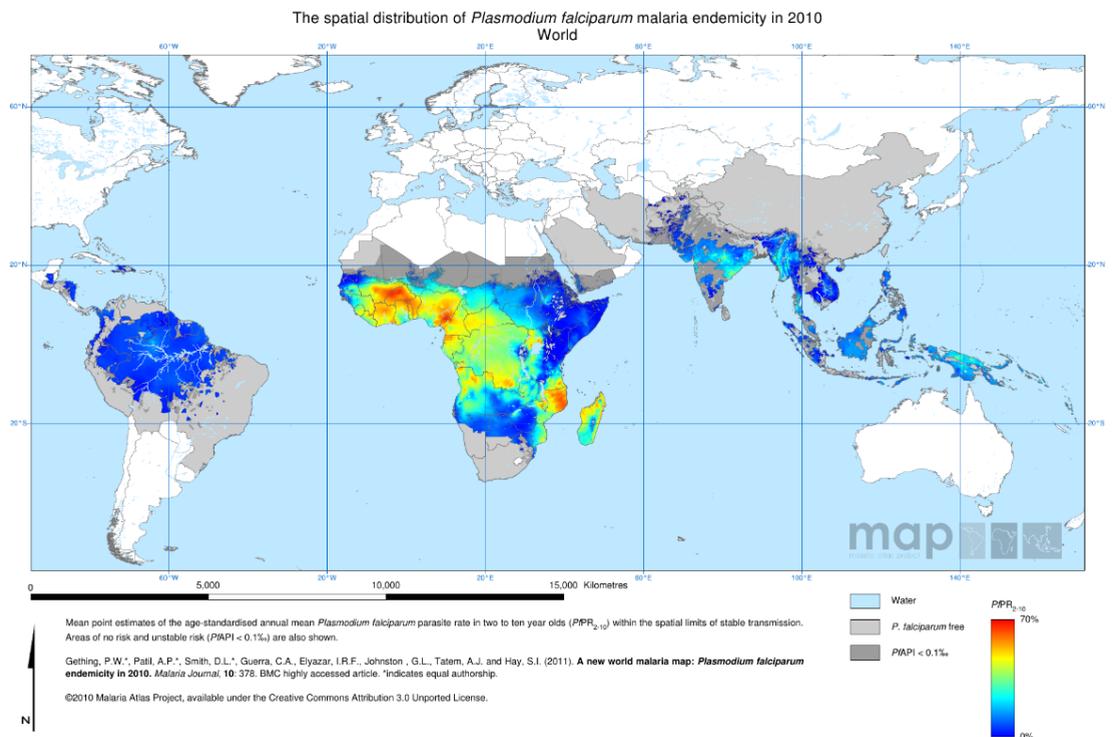


Figure 1. The spatial distribution of malaria endemicity in 2010 (adopted from the malaria atlas project; http://www.map.ox.ac.uk/browse-resources/endemicity/Pf_mean/world, accessed on April 1st 2014

1.2 THE MALARIA PARASITE AND LIFE CYCLE

Malaria is a protozoan parasite belonging to the genus *Plasmodium*. Five different species infect humans: *P. falciparum*, *P. vivax*, *P. ovale*, *P. malariae* and *P. knowlesi*. The latter was until recently only known to be naturally transmitted to macaques, but is now considered one of the human malaria species and transmission occurs in parts of South-East Asia (reviewed by Singh and Daneshvar 2013). A case report published in 2014 reported one case of naturally acquired infection with the simian malaria *P. cynomolgi* (Ta *et al* 2014), and the occurrence of zoonotic malaria in humans may be underestimated. Among the human malaria species *P. vivax* (Genton *et al* 2008, Tjitra *et al* 2008), as well as *P. knowlesi* (Cox-Singh *et al* 2013) can cause severe disease and death, however, most morbidity and mortality is attributed to *P. falciparum*.

This thesis will focus on *P. falciparum*, the deadliest and most widespread species of human malaria. The life cycle is complex and involves several distinct life stages both in the vector and the human host (Figure 2). The human infection starts with the bite of a parasite infected female *Anopheles* mosquito. Salivary glands of the mosquito contain sporozoite forms of the parasite (typically around 20) (Medica *et al* 2005, Rosenberg *et al* 1990) which are injected into the dermis together with anticoagulant saliva. Injected sporozoites will stay in the dermis for up to six hours (Yamauchi *et al* 2007) before entering the blood stream through which they are taken to the liver (Amino *et al* 2006), or as demonstrated in an animal model to a skin-draining lymph node (Amino *et al* 2006). The sporozoite migrates through the liver parenchyma before eventually invading one liver cell, creating a parasitophorous vacuole where it can differentiate and replicate asexually (schizogony) (Mota *et al* 2001). Each sporozoite gives rise to

tens of thousands of merozoites that are released into the circulation by budding from hepatocytes of parasite-filled vesicles called merozoites, after 6-15 days (Sturm *et al* 2006). Up to this stage, the infection is clinically silent.

Red blood cell invasion is a multi-step event where merozoites bind red blood cells through erythrocyte receptors and merozoite ligands (reviewed by Cowman *et al* 2012). Once inside the erythrocyte a protein trafficking system is built up in the infected red blood cell (RBC) by the parasite and its proteins are expressed on the surface of the host cell (Aikawa *et al* 1988). One of the best characterized families of these surface proteins is *P. falciparum* erythrocyte membrane protein-1 (PfEMP-1). PfEMP-1 mediates binding to endothelial cells via several receptors and allows sequestering of infected RBCs in various tissues (reviewed in Miller *et al* 2002). In parallel with these events the parasite differentiates and grows to ring- and trophozoite stages that can be visualized under light microscopy. This will be followed by asexual replication and the formation of thousands of merozoites that can be released into the blood stream and start yet another cycle of invasion. The replication cycle of *P. falciparum* takes approximately 48 hours, with an exponential increase in parasitemia (in non-immune individuals), exposing the host to increasing amounts of debris from the ruptured erythrocyte.

A small proportion of parasites will not form merozoites but develop to gametocytes, the sexual stage of the parasite that can be ingested with the blood meal of an anopheline mosquito (Bruce *et al* 1990). In the vector, male and female gametes replicate by meiotic recombination and form diploid zygotes that are able to develop into ookinetes that invade the gut epithelium. Cysts called oocysts then develop and thousands of sporozoites are released after rupture of the cyst and can invade the salivary glands (Touray *et al* 1992). Approximately

three weeks from the mosquito ingestion the parasites have matured and can once again infect the human host.

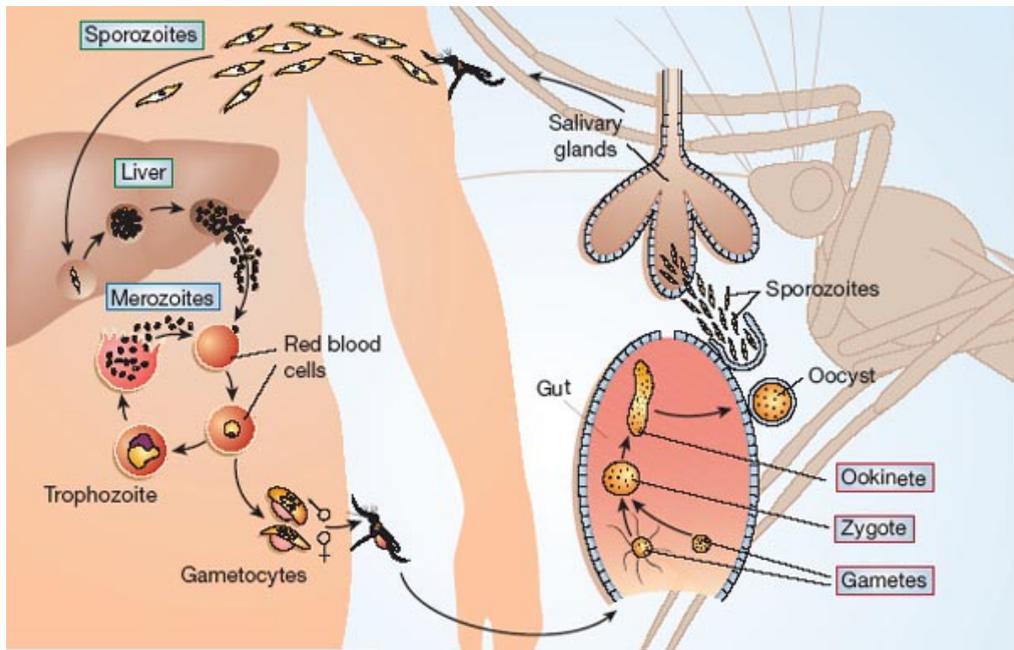


Figure 2. The life cycle of *Plasmodium falciparum* (Adopted from Ménard et al, Nature 2005)

1.3 MALARIA EPIDEMIOLOGY AND TRANSMISSION

Natural malaria transmission is restricted to areas where *Anopheles* mosquitoes are present and the climate is favorable for the parasite, i.e. tropical and sub-tropical areas.

Accurate monitoring of the malaria transmission is necessary for evaluation of interventions and control programmes against malaria, and endemicity of malaria can be monitored in several ways (reviewed by Tusting *et al* 2014)

The proportion parasite positive individuals by microscopy (parasite prevalence), or children with palpable spleens at a given time point are the most commonly used measurements for determination of level of malaria transmission (Gething *et al* 2011). Another widely used method is to determine the entomological inoculation rate (EIR), i.e. number of infected bites per person during a specified period of time. EIR is sensitive to seasonal variation and involves dissection of sporozoites after the catchment of mosquitos, where the gold standard is human landing catch (Onori and Grab 1980, Kilama *et al* 2014). With declining malaria transmission in many areas, more sensitive tools have become increasingly important for the measurement of transmission level. Polymerase chain reaction (PCR) has substantially improved the detection of parasites compared to microscopy (Okell *et al* 2012) and a number of polymorphic genetic markers of *P. falciparum* have been identified and can be used to define individual parasite populations (Contamin *et al* 1995). Rapid diagnostic tests (RDT) for detection of different malaria antigens are also useful for the detection of parasites in settings where submicroscopic infections are common (Stevenson *et al* 2013). Molecular force of infection (molFOI), the number of new parasite clones a host acquires per time unit, has good sensitivity for detection of changes in transmission, especially if a sensitive PCR method is used for the detection of parasite clones (Mueller *et al* 2012).

Transmission can be either endemic or epidemic. Endemic transmission is stable over several years, with or without seasonal fluctuations related to rain fall, in contrast to unstable or epidemic malaria transmission which features considerable fluctuations. Depending on intensity of transmission an area is considered hypoendemic (parasite prevalence by microscopy >10%), mesoendemic (11-50%), hyperendemic (50-75%) or holoendemic (>75%), according to the classical categorization (Metselaar and van Thiel 1959).

Malaria transmission can vary substantially within geographical areas, and spatial heterogeneity down to household level has been described (Greenwood *et al* 1989, Kruels *et al* 2008). Clusters with increased transmission are often referred to as hotspots (Bejon *et al* 2010).

1.4 CLINICAL PRESENTATION OF *PLASMODIUM FALCIPARUM* MALARIA

The clinical presentation of *P. falciparum* malaria ranges from benign and asymptomatic infections in the semi-immune host to severe disease and death in non-immune. Incubation time is at least seven days and commonly less than two months (Collins *et al* 1999, Schwartz *et al* 2003), although one case with 4 years from exposure to onset of symptoms has been reported (Greenwood *et al* 2008). The clinical manifestations of malaria are caused by asexual blood-stage parasites as they replicate in the blood.

1.4.1 Uncomplicated malaria

Fever is the most common symptom, usually spiking in an irregular pattern rather than corresponding to the 48 hour blood stage cycle of the parasite. Other symptoms include fatigue, nausea, chills, head ache, muscle ache, gastro-intestinal symptoms and cough. Intensity of symptoms is dependent on immune status of the host. Unless promptly treated, uncomplicated malaria can progress to severe malaria.

In endemic areas malaria and pneumonia share clinical features, and since both are common conditions in young children, differential diagnosis can be challenging (O'Dempsey *et al* 1993, English *et al* 1996, Källander *et al* 2004, Bassat *et al* 2011).

1.4.2 Severe malaria

Severe malaria is generally associated with a high risk of fatal outcome.

Mortality rate of 10.5% was reported in severe malaria cases imported to France (Bruneel *et al* 2010) and in African children mortality was 1.3-34.7% depending on syndrome (Marsh *et al* 1995).

Severe malaria is usually defined according to clinical and laboratory criteria set up by WHO (WHO 2000):

Clinical features:

- impaired consciousness or unrousable coma
- prostration, i.e. generalized weakness so that the patient is unable or sit up without assistance
- failure to feed
- multiple convulsions – more than two episodes in 24 h
- deep breathing, respiratory distress (acidotic breathing)
- circulatory collapse or shock, systolic blood pressure < 70 mm and < 50 mm Hg in children
- clinical jaundice plus evidence of other vital organ dysfunction
- haemoglobinuria
- abnormal spontaneous bleeding

-pulmonary oedema

Laboratory findings:

-hypoglycaemia (blood glucose < 2.2 mmol/l or < 40 mg/dl)

-metabolic acidosis (plasma bicarbonate < 15 mmol/l)

-severe normocytic anemia (Hb < 5 g/dl)

-haemoglobinuria

-hyperparasitaemia (> 2% infected erythrocytes or 100 000 parasites/ μ l in low intensity transmission and non-immune individuals or > 5% or 250 000/ μ l in areas of high stable malaria transmission intensity)

-hyperlactataemia (lactate > 5 mmol/l)

-renal impairment (serum creatinine > 265 μ mol/l)

Severe pediatric malaria typically features three major and often overlapping syndromes:

- A. Cerebral malaria is clinically defined as unrousable coma in patients with *P. falciparum* parasitemia (Molyneux *et al* 1989). White blood cell count in cerebrospinal fluid is usually <10 cells/ml in cerebral malaria cases (Berkley *et al* 1999). Impaired consciousness is a prognostic indicator of death in children with severe malaria (Marsh *et al* 1995) and neurological sequelae are common among survivors (Idro *et al* 2008, Birbeck *et al* 2010).
- B. Severe malarial anemia (Hb <5 g/dl), usually affects children < 3 years of age (Obonyo *et al* 2007)

C. Respiratory distress, characterized by deep acidotic breathing as a marker of acidosis and an indicator of severity (English *et al* 1996). Deep breathing has been shown to be a good “proxy” for metabolic acidosis (Marsh *et al* 1995).

Peak incidence of cerebral malaria in endemic areas is in children >2 years old (Gupta *et al* 1999). As transmission intensity declines a greater proportion of severe malaria admissions will be in older children, and cerebral malaria will account for a larger proportion of severe malaria admissions. No relationship between the proportions of children with severe malaria anaemia and transmission intensity has been shown (Okiro *et al* 2009, Carneiro *et al* 2010).

Studies in endemic countries show that malaria strongly predisposes children to bacteremia and invasive bacterial infections are considered a complication to *P. falciparum* infections (Scott *et al* 2011). Bacteremia incidence is particularly high in severe malaria cases (Bronzan *et al* 2007, Bassat *et al* 2009, reviewed in Church *et al* 2014).

1.4.3 Malaria in travelers

Travel to malaria endemic countries is increasing and despite being treatable and preventable imported malaria carries a mortality rate of around 1% according to reports from high income countries (Checkley *et al* 2012, Newman *et al* 2004). Risk factors associated with death from imported malaria include older age, European origin, travel to east Africa, absence of chemoprophylaxis, hyperparasitemia, and delay in seeking medical care and delayed diagnosis and treatment of suspected malaria (Newman *et al* 2004, Legros *et al* 2007, Bruneel *et al* 2010).

Travelers with malaria often present with cough (Wyss unpublished 2014), and can be mistaken for a respiratory tract infection if the clinician attending is not aware of the diagnosis.

Antibiotic treatment is often initiated alongside anti-malarial treatment in travelers diagnosed with malaria. Invasive bacterial infections were however found to be uncommon in patients treated for malaria in Sweden (Sandlund *et al* 2013).

1.5 NATURALLY ACQUIRED IMMUNITY TO MALARIA

1.5.1 Acquisition of immunity to malaria

Individuals in malaria endemic areas gradually develop protective immunity after repeated exposure to the parasite (Marsh *et al* 2006). In areas of high transmission, immunity to severe forms of malaria develops within the first years of life, whilst immunity to mild disease is acquired in older children or during adolescence. Immunity is never sterilizing, but rather confers protection against high parasite densities and symptoms (Figure 3).

Infants are protected during approximately the first six months of life, and during this period malaria infections are often self-limiting and with low parasite densities. This protection has been associated with both fetal Hb and passive transfer of maternal anti-malarial IgG (Amaratunga *et al* 2011).

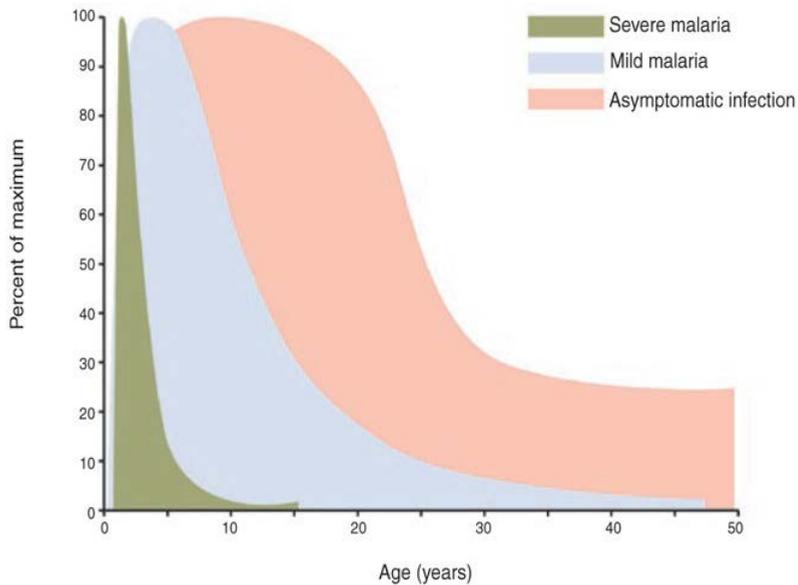


Figure 3. Change over time of various indices of malaria in a population living in an endemic area of *P. falciparum* transmission: asymptomatic infection (pink), mild disease (febrile episodes caused by malaria; blue) and severe or life-threatening disease (green). The data are normalized and are presented as the percent of maximum cases for each population index (Adopted from Langhorne et al Nature Immunology 2008 and printed with permission from Nature Publishing Group)

The relatively inefficient way that immunity to malaria develops has been attributed in part to the genetic diversity of many *P. falciparum* proteins (reviewed in Takala *et al* 2009) as well as the antigenic variation the parasite exhibits during infection (Scherf *et al* 2008). For the polymorphic merozoite antigens, antibody responses to individual antigens have in some studies been associated with protection whereas others failed to demonstrate this effect

(Fowkes *et al* 2010) However, breadth (the number of antigens recognized by an individual) and magnitude of antibody responses have been associated with protection from malaria (Osier *et al* 2008) and a possible explanation for the conflicting results from many immuno-epidemiological studies is that antibody levels above a certain threshold are required for the protective effect (Murungi *et al* 2013).

The presence of asymptomatic parasitemia has been associated both with a reduced risk of clinical malaria (Crompton *et al* 2008, Males *et al* 2008) as well as higher levels and increasing breadth of anti-merozoite antibody levels (Osier *et al* 2008, Rono *et al* 2013), suggesting that asymptomatic low-grade infections contribute to protection against clinical malaria. Repeated malaria episodes did on the other hand not contribute to an increase in anti-merozoite antibody levels in Kenyan children in a low endemic area who experienced multiple episodes (Rono unpublished 2014).

Immunity is at least in part strain-specific, as demonstrated in patients with neurosyphilis who were treated with successive malaria inoculations to cause fever. When the patients were infected with homologous *P. falciparum* strains lower parasitemia and less fever was induced as compared to if infected with heterologous strains over successive events (Jeffery *et al* 1966).

Studies from Indonesia showed that in migrants, who arrived to an area of holoendemic transmission, protective immunity was quicker to develop in adults compared to children and the degree of protection was related to age and recent exposure (Baird *et al* 1991). This suggests that factors other than exposure and possibly intrinsic features related to age are important for the acquisition of immunity.

1.5.2 Maintenance of protective immune responses

Immunological memory is characterized by the ability to recognize previously encountered antigens and react with a specific and more rapid response compared to at the primary infection. A long-lived memory is dependent on the generation of long-lived plasma-cells (LLPC) and MBCs. Naïve B-cells are activated upon encounter with foreign antigen and differentiate into either antibody-producing short-lived plasmablasts or resting MBC or antibody-producing LLPC, each clone specific for one defined antigen. LLPCs reside in the bone marrow and produce antibody continuously, whereas MBC circulate and are ready to proliferate rapidly upon secondary exposure to their respective antigen (Gourley *et al* 2004).

The clinical protection against malaria has anecdotally been said to wane shortly after ceased exposure (Colbourne *et al* 1955). This is also indicated by the epidemiology of malaria in travelers, where immigrants in malaria-free countries who were previously highly exposed during childhood succumb to malaria after visiting relatives (Bochaud *et al* 2005). However, when malaria re-appeared epidemically in Madagascar after decades of successful control, people present 30 years earlier during continuous transmission were more resistant to clinical disease compared to younger subjects (Deloron and Chougnet 1992) and immigrants living in France for at least four years had lower parasite densities, shorter time with fever and lower frequency of complicated disease compared to French natives admitted with malaria in France (Bochaud *et al* 2005). Few studies report on which components of the immune responses that are lost, and especially in total absence of malaria exposure. Antibodies against malaria antigens are often short-lived (Kinyanjui *et al* 2007, Akpogheneta *et al* 2008,

Akpogheneta *et al* 2010, White *et al* 2014) (especially in children), in contrast to many other infectious diseases (Amanna *et al* 2007).

Maintenance of durable antibody levels sufficient to provide protection over time, and the ability to rapidly respond upon re-exposure to a previously encountered antigen is dependent on the generation of MBCs and long-lived plasma cells (LLPC). Malaria specific MBCs appear to be more long-lasting than antibodies, even with absent or low exposure (Wipasa *et al* 2010, Ndungu *et al* 2012, Ampomah *et al* 2014) but are generated stepwise and with repeated malaria exposure (Weiss *et al* 2010). An association with increased breadth of MBC responses with increasing age and exposure has been reported (Nogaro *et al* 2011). Malaria specific MBCs are however only found in a proportion of exposed individuals (Wipasa *et al* 2010, Weiss *et al* 2010), and this relatively inefficient way of generating immunological memory is likely to contribute to the long duration needed to acquire protective levels and/or breadth of antibody responses.

Studies in individuals living in malaria areas have identified an increase in proportion of a phenotypically distinct subset of MBCs, often referred to as “atypical” memory B-cells (Weiss *et al* 2009, Illingworth *et al* 2013). A similar pool of cells has been found to be expanded in HIV viremic patients compared to healthy controls (Moir *et al* 2008). The observations of atypical MBC subsets contracting during absence of exposure (Ayieko *et al* 2013, Weiss *et al* 2009) may indicate that there is a causal link between malaria exposure and this expanded cell pool. In Mali, a trend towards a higher proportion of atypical MBCs in individuals asymptotically infected through the dry season was observed (Weiss *et al* 2009). Atypical MBCs from immune donors have been shown *in vitro* to actively produce antibodies with neutralizing effect on blood

stages of *P. falciparum* (Muellenbeck *et al* 2013). Further studies of specificities and association with protection of MBCs are warranted, as well as elucidation of links between asymptomatic parasitemia and expansion of the atypical MBC compartment.

1.6 MALARIA VACCINES

A malaria vaccine is likely to have a major impact on global health. Since the burden of disease is extensive, even a vaccine with only partial efficacy would be of great importance. Current approaches involve development of vaccines based on immunization with whole parasites and against different life stages of the parasite as well as specific mechanisms, such as pregnancy associated malaria or pathogenic mechanisms of severe malaria. Below is a brief introduction to the vaccines under development aiming at targeting pre-erythrocytic stages, asexual blood stages or the sexual stages blocking transmission to the mosquito (reviewed by Crompton *et al* 2010).

1.6.1 Pre-erythrocytic vaccines

A strong argument for a malaria vaccine targeting the pre-erythrocytic phase of malaria infection are studies showing that inoculation with irradiated *P. falciparum* sporozoites induce durable and strain-transcending protection in healthy volunteers (Clyde *et al* 1973, Hoffman *et al* 2002).

The most successful malaria vaccine until now, RTS,S vaccine, has recently undergone phase III trials in Sub-Saharan Africa. The RTS,S, vaccine consists of a subunit of the circumsporozoite surface protein fused to hepatitis B surface proteins and the adjuvant AS01. After one year follow-up, results showed that three doses of RTS,S reduced clinical malaria by 56% in children 5-17 months of age at first vaccination (Agnandji *et al* 2011). In infants 6-12 weeks of age at

first vaccination with RTS,S, clinical malaria was reduced by 31% (Agnandji *et al* 2012).

1.6.2 Asexual blood stage vaccines

Pathology from malaria infection develops in the asexual blood stage of infection, making this an argument for the development of a vaccine targeting antigens involved in either invasion or adhesion of infected red blood cells. Such a vaccine would prevent symptoms but not infection, similar to the situation where immunity is developed naturally.

Antibodies are thought to play a key role in controlling parasite densities and pathology during blood stage infection in humans (Cohen *et al* 1961, Sabchareon *et al* 1991), further supporting the development of blood-stage vaccines.

A number of merozoite antigens are under current investigation as vaccine candidates, and several vaccines have undergone phase II trials. Antigens being investigated are of importance for proliferation of the parasite and naturally acquired antibodies correlate well with protection in many immunological studies (Polley *et al* 2003, Fowkes *et al* 2010). Focus has mainly been on MSP-1 AMA-1, MSP-2, MSP-3 and EBA-175 (Ogutu *et al* 2009, Otsyula *et al* 2013, Malkin *et al* 2008). To date, none of these antigens have alone shown to confer protection in phase II trials (reviewed in Goodman and Draper 2010). Vaccines combining different antigens have also been investigated in phase I and II trials, but so far only RTS,S has undergone phase III trials.

Several studies provide evidence that antigens expressed on the surface of the infected red blood cell are involved in pathophysiology through adhesion mechanisms. Special focus has been given to the PfEMP-1 family of proteins, where certain variants of the proteins have been associated with severe malaria

and mechanisms such as rosetting and adhesion of infected red blood cells to vascular endothelium (Treutiger *et al* 1997, Heddini *et al* 2001, reviewed by Craig and Scherf 2001). These antigens are targets for naturally acquired immunity to malaria (Bull *et al* 1998). The variant VAR2CSA mediates adhesion to syncytiotrophoblasts in the placenta (Fried and Duffy 1996) and protective immunity to these parasite variants develops over successive pregnancies (reviewed in Hviid 2004). The extensive polymorphism of the PfEMP1 family of proteins is a challenge in vaccine development; however antibodies targeting conserved regions or functional receptor binding sites may block this adhesion and may yet prove to be successful against the variants that cause placental malaria (Salanti *et al* 2013) and other adhesion phenotypes (Berger *et al* 2013).

1.6.3 Transmission blocking vaccines

Antibodies against sexual stages are elicited during natural *P. falciparum* infection (Ouedraogo *et al* 2011), and this together with the opportunity to reduce transmission on a population level provides the strongest argument for the development of a vaccine against gametocytes. A transmission blocking vaccine raises ethical considerations since it does not provide any protection from disease for the individual vaccinated, and this type of vaccine will most likely be co-formulated with antigens that induce protection also against other stages of the malaria parasite's life cycle.

1.7 P. FALCIPARUM MEROZOITE ANTIGENS

Merozoite antigens were selected for the purpose of the studies in this thesis based on their immunogenicity and/or since they have potential functional relevance and are under development as vaccine candidates.

1.7.1 Merozoite surface protein-1

MSP-1 is believed to be involved in the initial attachment of the merozoite to the erythrocyte and is present in abundance on the merozoite surface (O'Donnell *et al* 2000, O'Donnell *et al* 2001). Full length MSP1 is initially 190 kDa, but is subsequently cleaved multiple times so that by the time of erythrocyte invasion only the C-terminal 19 kDa fragment (often referred to as MSP-1₁₉) is present on the merozoite surface. The sequence of full length *msp1* has 17 blocks and can be grouped into major allelic types; based on polymorphisms in block 2 the three allelic types MAD-20, K1 and RO33 are described (Tanabe *et al* 1987, Takala *et al* 2002).

1.7.2 Merozoite surface protein-2

MSP-2 is a 30 kDa intrinsically unstructured protein anchored in the plasma membrane of the merozoite (Smythe *et al* 1988) and has a tendency to form fibrils in vitro (Yang *et al* 2010). The exact function of MSP-2 has not been established; however the extensive genetic polymorphism of its sequence indicates that it has been under selection pressure from human immune responses. The *msp2* gene can be grouped into 5 blocks based on sequence type (Snewin *et al* 1991). Block 1 and 5 are highly conserved, block 2 and 4 semi-conserved and the central block 3 region is highly variable with conserved regions flanking regions with repetitive units with different length and copy numbers. *Msp2* can be grouped into two major allelic types: IC (also referred to as 3D7) and FC-27, based on the non-repetitive sequences that flank the polymorphic region present in block 3 (Smythe *et al* 1991).

1.7.3 Merozoite surface protein–3

MSP-3 is expressed in the schizont stages and after cleavage parts of the protein will be present at the merozoite surface (McColl and Anders 1997). The polymorphic N terminal sequence defines the two major allelic types of *msp3* (3D7 and K1, Huber *et al* 1997).

1.7.4 Apical membrane antigen–1

AMA-1 is an 83kDa protein that mediates apical re-orientation in the final phase of the invasion process of the merozoite. The gene encoding AMA-1 is highly polymorphic and has been under selection pressure by host immunity (Polley and Conway 2001). Polymorphisms are mainly due to SNPs (McColl *et al* 1997).

1.8 MOLECULAR EPIDEMIOLOGY OF *P. FALCIPARUM* MALARIA

The extensive genetic diversity of many *P. falciparum* antigens is likely to contribute to the long time it takes to develop immunity to malaria, and is one of the challenges that needs to be overcome in the design of a malaria vaccine.

The introduction of PCR genotyping in malaria research has contributed significantly to knowledge of several aspects of parasite biology and epidemiology. The most widely implemented genetic markers are *msp1*, *msp2* and *GLURP* which have been used both in drug trials to distinguish reinfection from recrudescence and for characterization of parasite populations in molecular epidemiology studies (Zwetyenga *et al* 1998, WHO 2007). Among these *msp2* is the most informative single marker (Färnert *et al* 2001).

Asymptomatic infections are often of low parasite densities, and optimal sampling, storage and DNA extraction methods are of importance for sensitive and reproducible results (Färnert *et al* 1999).

1.8.1 Factors influencing infection diversity

Plasmodium falciparum infections are often composed of several concurrent parasite clones, resulting either from a bite from a mosquito infected with several genetically different sporozoites (Druilhe *et al* 1998, Arez *et al* 2003) or as a result of superinfection from successive inoculations. In areas with low transmission or at epidemic outbreaks of malaria, the number of clones within individuals and totally in the population is generally lower (Tami *et al* 2002, Mueller *et al* 2002) compared to areas of high transmission where meiotic recombination in the mosquito will take place more often, generating new and more allelic variants (Walliker *et al* 1983). Increasing transmission is associated with a non-linear increase in the average number of clones within an individual (Bendixen *et al* 2001), and up to 12 individual clones present at the same time has been reported (Konate *et al* 1999).

Studies reporting on infection diversity at the time of clinical malaria report conflicting results with regards to number of clones within the course of an acute malaria episode compared to asymptomatic infection, with some studies reporting parasite densities to have a positive correlation with number of clones detected (Smith *et al* 1999), or the number of clones have been described to be higher in individuals with symptoms (Zwetyenga *et al* 1998). One study where genetic diversity was assessed by *m*sp2 indicated the lowest number of clones in fatal cerebral malaria and asymptotically infected individuals, whereas uncomplicated malaria and severe malaria anemia had higher number of clones (Al el Basit *et al* 2007).

Clearance of parasites by treatment and the prophylactic effect of long-acting anti-malarial drugs in the blood will also decrease the number of circulating clones (Liljander *et al* 2010, 2011). The effect of more frequent treatment in young and less immune children is likely to contribute to the decrease in the number of detected clones compared to older age groups.

Some previous studies have reported a higher number of clones within individuals with sickle cell trait (Ntoumi *et al* 1997, Kiwanuka *et al* 2009), whereas others have not found such differences (Vafa *et al* 2008, Koukouikila-Koussounda *et al* 2012).

Age is associated with diversity of infections in high transmission areas with the peak in mean number of clones at the age of 3-10 years, co-inciding with the development of protective immunity (Ntoumi *et al* 1995, Engelbrecht *et al* 1995, Konate *et al* 1999, Bendixen *et al* 2001). In areas of low transmission no difference in the mean number of clones is observed between age groups (Zwetyenga *et al* 1998, Konate *et al* 1999). The decrease of diversity observed during adulthood reflects the development of immunity and with lower parasite densities some clones might not be detected. Individual exposure to mosquito bites is likely to affect infection diversity; however studies on the effect of bednet use on number of clones have not provided evidence of such an effect (Fraser Hurt *et al* 1999, Smith *et al* 1999).

Accumulation of multiple parasite clones takes several years also in highly endemic settings. One mechanism identified in rodent malaria models; where blood stage infection above a certain density threshold prevents liver stage superinfection to be established (Portugal *et al* 2011). Younger children with less immunity to blood stages and thus higher parasite densities will be superinfected less commonly, and with older age when blood stage immunity is potent enough

to suppress higher parasite densities this will provide an opportunity for novel parasite clones to be established.

1.8.2 Infection dynamics

Asymptomatic blood stage parasites can persist within an individual for several months (or more) (Franks *et al* 2001). Duration of infections appears to be affected by both transmission intensity and age (Smith *et al* 1999) with peak in average duration of infections in children 5-9 years of age (200-300 days depending on model used) (Felger *et al* 2012). Since parasites intermittently disappear from circulation (likely due to sequestration in tissues) single time point measurements provide only part of the patterns of infecting parasites (Färnert *et al* 1997, Daubersies *et al* 1996). Infections during a febrile malaria episode appear to confer less synchronised pattern with the same genotypes being detected in consecutive samples (Färnert *et al* 2005, Carlsson *et al* 2011).

1.8.3 Infection diversity and risk of malaria

The number of clones in an asymptomatic host and association with subsequent risk of clinical malaria has been investigated in several studies.

In areas of high transmission, a reduced risk of febrile malaria was associated with carriage of multiclonal infections, both in 6-16 year old children followed for 40 weeks (Bereczky *et al* 2007) as well as 1-10 year old children followed during four years (Färnert *et al* 1999) in Tanzania, and 1-16 year old children in a high transmission setting in Papua New Guinea (Al-Yaman *et al* 1997). On the other hand, in children below three years of age the presence of multiclonal infections has been associated with an increased risk of malaria (Henning 2004, Branch 2001). In settings with seasonal (Roper *et al* 1997, Babiker *et al* 1998, Ofose-Okoyere *et al* 2001,) and holoendemic (Kun *et al* 2002) malaria

transmission, symptoms of febrile malaria have been associated with the introduction of a clone that is new to the host. In one study of 1-3 year old children in Papua New Guinea, infection with new clones (force of infection, FOI) was a major factor associated with the risk of clinical malaria (Mueller *et al* 2012).

In some settings and age groups, a high number of clones in an asymptomatic host might reflect the ability of the host to harbor parasites with an antigenically diverse repertoire and acquired immunity. In younger age groups or when transmission is increasing or unstable the higher number of clones might be more the result of recent inoculations in a host with a lower degree of immunity and a larger proportion of infections will develop into symptom-causing malaria.

Understanding of the importance of exposure to genetically diverse asymptomatic infections for the development and maintenance of protective immunity is still incomplete and impact of duration of infections and number of infections needed to achieve protection against severe malaria needs to be further clarified.

2 AIMS OF THESIS

The overall aim of this thesis was to contribute to the understanding of the acquisition and maintenance of immunity to malaria, especially in relation to exposure to genetically diverse infections and malaria specific immunological memory.

The specific aims in the included papers were:

- I. To investigate whether the pattern of exposure; particularly number of infections and exposure to different clones in early childhood, affect the risk of severe malaria
- II. To improve DNA extraction methodology for PCR from frozen residual blood clots (to resolve methodological issues in paper I)
- III. To describe the epidemiology of *P. falciparum* diversity in an area of seasonal malaria transmission and to assess whether the genetic diversity of *P. falciparum* infections persisting through the dry season affects the risk of clinical malaria at the following transmission season
- IV. To investigate the longevity of malaria-specific immune responses in absence of re-exposure in travelers treated for malaria in a non-endemic setting

3 MATERIAL AND METHODS

3.1 ETHICAL CONSIDERATIONS

All studies were granted ethical approval by the Regional Ethical Review Board in Stockholm, Sweden. For study I and II an ethical permit was also obtained from the Kenyan Medical Research Institute National Ethics Committee. The study was explained to parents or guardians by team members fluent in Swahili or Giriama and parents were also given written versions of the information. For study III ethical approval was granted by the Faculty of Medicine, Pharmacy and Odonto-Stomatology Ethics Committee; and the Institutional Review Board at the National Institute of Allergy and Infectious Diseases, National Institutes of Health. Written, informed consent was obtained from adult participants, and from the parents or guardians of participating children. For study IV, the study concept was explained orally and in text and written informed consent was obtained from study participants.

3.2 STUDY POPULATIONS AND CASE DEFINITIONS

3.2.1 Kenya (study I and II)

Study I and II were conducted in Kilifi District on the Kenyan Coast, where transmission intensity varies between locations but has generally decreased since 2000. A large birth cohort, Kilifi Birth Cohort (KBC) was set up in 2001 to study the immuno-epidemiology of pneumococci (Scott *et al* 2012). Children either born at Kilifi District Hospital or presenting to the vaccination clinic within their first month of life were recruited and subsequently followed with three-monthly visits during their first two years of life. At each visit axillary temperature was measured together with collection of 2 ml of blood and a malaria slide. Births and migrations were monitored through the Kilifi Health and Demographic

Surveillance System (KHDSS) and all hospital admissions to Kilifi District Hospital were recorded, allowing for cross-checking between data bases for identification of severe malaria cases within the birth cohort.

A matched case-control study was nested within the larger KBC. Severe malaria cases were classed as children with *P. falciparum* parasitemia together with one or more of the three major severe malaria syndromes: (1) impaired consciousness defined as Blantyre Coma Score <5; (2) severe anemia defined as hemoglobin <5g/dl; and/or (3) deep breathing and/or chest indrawing i.e. respiratory distress as a sign of acidosis. Children with positive bacterial cultures from blood or cerebrospinal fluid and/or >10 white blood cells in cerebrospinal fluid were excluded to avoid misclassification of children with other infections and incidental parasitemia. Controls were children in KBC who were not admitted with severe malaria and selected based on matching to the cases for area of residency, age and time of sampling.

3.2.2 Mali (study III)

The study was carried out in Kambila, a small rural village, situated 20 kilometers north of Bamako, the capital of Mali. Transmission of *P. falciparum* is seasonal and intense, peaking in September through October and ending in December. 225 individuals from an age-stratified random sample were included at the baseline survey in May 2006, just before the malaria transmission season started. The study cohort consisted of four pre-defined age groups (2-4, 5-7, 8-10 and 18-25 years) and cross-sectional surveys were performed bi-monthly during the malaria transmission season (July, October and December 2006) and prior to the second malaria season. During the one-year study period, cohort members were encouraged to report signs and symptoms of malaria to a study physician in the village who was present 24 hours per day, 7 days per week. At every un-

scheduled “sick-visit” microscopy for malaria was performed and if parasites were detected a standard three-day course of artesunate plus amodiaquine was given according to the national guidelines in Mali. The more specific definition of clinical malaria used in the analyses was an axillary temperature $\geq 37.5^{\circ}\text{C}$, *P. falciparum* asexual parasitemia $\geq 5000/\mu\text{l}$, and a non-focal physical exam by the study physician.

3.2.3 Sweden (study IV)

Individuals who had been admitted to hospital in Stockholm, Sweden, with *P. falciparum* malaria between 1994 and 2010 were invited to participate in the study. Among 270 invitees, 47 consented to participate and were sampled once during May 2011. The median time between their malaria admission and the cross-sectional survey was 11 years (range 1-17). Of the study participants, 33 were born in Europe and 12 in countries endemic for *P. falciparum*.

For comparison the study also included 8 Swedish adults with no prior exposure to malaria and 14 adults who originated from and were resident in an area of high transmission in Junju, Kenya.

3.3 GENOTYPING OF *P.FALCIPARUM*

3.3.1 DNA extraction

In study I, DNA was extracted from frozen residual blood clots retrieved when preparing serum and thus stored without anticoagulant according to the methods developed in study II. Briefly, blood clots were processed with high speed shaking followed by chemical extraction with Puregene commercial kits (Qiagen) according to the procedure described in detail in study II.

In study III DNA was extracted with Qiagen Blood Mini Kit (Qiagen) from dried blood spots collected on filterpaper 4-5 years prior to extraction.

3.3.2 *Msp2* genotyping PCR

Genotyping of *P. falciparum* was performed using a two-step, nested PCR method, with a modified protocol adopted from Snounou (Snounou 1999, Liljander 2009). In the primary reaction the entire block 3 of *msp-2* is amplified. In the second PCR reaction amplified material from the first reaction is used as template in two separate reactions with fluorescently labeled primer pairs targeting the respective allelic types of *msp-2*: FC27 and IC (also referred to as 3D7). The forward primers in the nested reaction were tailed with a 7-bp tail (Applied Biosystems) at the 5'-end in order to promote the non-template adenosine (A) addition by the Taq DNA polymerase at the 3' end of the PCR products, and reverse primers were tagged with a fluorophore (Table1)

Table 1. Primer sequences and fluorescent markers for *msp2* PCR

Oligonucleotide primers in the primary reaction		Fluorescent marker
<i>msp2</i> F	5'-ATGAAGGTAATTTAAAACATT GTCTATTATA-3'	-
<i>msp2</i> R	5'- CTTTGTACCATCGGTACATTCTT-3'	-
Allelic type-specific oligonucleotide primers in the nested reaction		
FC27 F	5'- AATACTAAGAGTGTAGGTGCARATGCTCCA-3'	7 bp-tail
FC27 R	5'- TTTTAT TTG GTGCAT TGCCAGAAC TTG AAC-3'	6-FAM™ (blue)
ICF	5'- AGAAGTATGGCAGAAAGTAAKCCTYCTACT-3'	7 bp-tail
ICR	5'- GATTGTAATTCGGGGGATTCAGTTTGTTTCG-3'	VIC® (green)

All PCR reactions were performed in 96-well plates and with 2 μ l DNA template and 20 μ l total reaction volume. Positive DNA controls in different concentrations for assessment of the sensitivity of the reaction and negative controls were added to each plate.

In the primary reaction final concentration of the mastermix consisted of 1xPCR buffer, 2mM MgCl₂, 125 μ M dNTP, 0.02 units/ μ l AmpliTaq DNA polymerase (Applied Biosystems). The cycle conditions were: Initial denaturation at 95°C for 5 min followed by 25 cycles of annealing at 58°C for 2 min, extension at 72°C for 2 min, denaturation at 94°C for 1 min with a final round of 58°C for 2 min and 72°C for 5 min.

In the second reaction one μ l of amplified material from the first reaction was used as template and final concentration of the master mix consisted of 1 \times PCR buffer, 1 mM MgCl₂, 125 μ M dNTP and 0.02 units/ μ l polymerase in the *msp2* FC27 reaction and 0.05 units/ μ l of AmpliTaq DNA, and 125 nM of the *msp2* FC27 primers and 250 nM of the IC type primers in separate reactions. Cycle conditions were: Initial denaturation at 95°C for 5 min, followed by 23 cycles of annealing at 58°C for 1 min, extension at 72°C for 1 min followed by 94°C for 30 sec, and a final round at 58°C for 1 min and 5 min at 72°C.

Separation of fluorescent fragments was performed by capillary electrophoresis in a 3730 DNA sequencer (Applied Biosystems). Products were diluted 1:10 to decrease background artifacts and 1 μ l diluted product was added to 9 μ l Hi-Di formamide together with 0.25-0.5 μ l size standard (GS-LIZ1200, Applied Biosystems). To avoid inclusion of background in the true results a fluorescent cut-off of 150-300 rfu was applied. Peaks corresponding to amplified alleles

were analyzed with GeneMapper software (Applied Biosystems) but also visually scanned for quality control. Since the method varies 0.5-1 base pairs between runs a three base pair binning system was implemented to distinguish individual allelic variants of *msp2*.

3.4 QUANTIFICATION OF ANTI-MALARIAL ANTIBODIES

For study I and IV antibodies against *P. falciparum* parasite lysate (schizont extract) were quantified by enzyme-linked immunosorbent assay (ELISA). Preparation of parasite extract and ELISA was performed according to a previously described protocol (Ndungu *et al* 2002).

For study IV, antibodies against recombinant proteins expressed in *E. coli* were kindly provided by Louis Miller and Susan Pierce at NIH. Full-length AMA-1 from the 3D7 and FVO alleles respectively were mixed in a 1:1 ratio before coating of ELISA plates. MSP-1 was expressed as the 42 kDa fragment and MSP-3 in full length.

3.5 ISOLATION OF PMBC

In study IV, PBMCs were isolated from 30 ml of heparinized blood per donor. Blood was mixed with an equal volume of RPMI and this mixture was layered on Ficoll-Paque and centrifuged. The PBMC layer was collected and washed twice in RPMI before the addition of FCS and DMSO prior to freezing in aliquots of 1 ml cell suspension per vial.

3.6 QUANTIFICATION OF MALARIA-SPECIFIC MEMORY B-CELLS

Samples were thawed and counted before dilution to desired cell concentrations. Memory B-cells were quantified using a modified ELISpot protocol (Weiss *et al* 2012) adopted from a previously developed assay (Crotty *et al* 2004). Briefly, PBMCs are cultured for 5 days together with a stimulant mastermix containing CpG oligonucleotide (Operon), Protein A from *Staphylococcus aureus* Cowan strain (Sigma-Aldrich), pokeweed mitogen (Sigma-Aldrich) and IL-10 (R&D systems). ELISpot plates (Millipore Multiscreen HA) were pre-coated with malaria antigens AMA-1 (FVO and 3D7 alleles in 1:1 mixture), MSP-1₄₂ or MSP-3, polyclonal goat antibody specific for human Ig-G (for the quantification of total Ig-G secreting cells), bovine serum albumin (used as a negative control to correct for unspecific binding in the plates) or tetanus toxoid (as a positive control). Cultured cells were added to the plates in triplicate and incubated for 5 hours at 37°C in 5% CO₂. Plates were then washed and further incubated with alkaline phosphatase-conjugated goat anti-human IgG Fc antibodies. Plates were washed seven times before being developed overnight in the dark and spots were counted using an ImmunoSpot series 4 analyzer (Cellular Technologies LTD) and results analyzed using ImmunoSpot version 5 software (CTL). In addition, a manual inspection of spots was also performed in all samples as quality control. The results were then reported as frequencies of MBC's per 10⁶ PBMCs.

3.7 PHENOTYPING OF B-CELL SUBSETS

In study IV, B-cell subsets were characterized by flow cytometry (Beckman Coulter CyAn ADP) after cell surface staining with a panel of fluorophore-labelled monoclonal antibodies as described previously (Weiss *et al* 2009). Data was analyzed with FlowJo Software (Tree Star, USA).

3.8 STATISTICAL METHODS

Data analyses were performed in Stata version 11 and GraphPad PRISM version 5. R version 2.13.1 was used in study I.

In study I a conditional logistic regression was performed, including risk sets with at least one matched control available. The analysis was adjusted for the logarithm of the number of visits. Furthermore, an unconditional case-control study was performed within cases with different syndromes, using an exact logistic regression with adjustment for age and number of visits.

In study III Cox regression (Cox 1972) was made as well as risk assessment with Andersen-Gill regression model (AG model), under which the risk of an event is unaffected by the occurrence of earlier events and multiple malaria episodes within one individual can be accounted for. Since some individuals are more prone to infections than others we included a gamma-distributed frailty term in the AG model (Andersen and Gill 1982).

In study IV, antibody and MBC data was log-transformed and analyzed using Stata version 11 (Stata Corp, USA) and GraphPad Prism for Macintosh version 5.0 (GraphPad Software, USA). Multivariable linear regression analysis was used to test the predictive value for origin of the travelers (defined by birth in either countries where malaria is endemic or nonendemic), time since malaria diagnosis, age at admission with malaria, parasitemia at diagnosis, previous episodes, and duration of symptoms before cure. Kruskal–Wallis test (with Dunn’s correction for multiple comparisons) was used to compare continuous variables between groups. For all tests, two-tailed p-values were considered significant if $p < 0.05$.

4 RESULTS

4.1 STUDY I:

***Plasmodium falciparum* infection patterns since birth and risk of severe malaria: a nested case-control study in children on the coast of Kenya**

The aim was to study whether exposure patterns early in life affects the risk of developing severe malaria. A case-control study was nested within a birth cohort study including 5949 children. Among the cohort participants 61 children had been admitted to Kilifi District Hospital with severe malaria according to strict case definition. Community controls selected within the cohort (n=161), were matched on age, area of residency and time of sampling. The median age at the severe malaria admission was 14.8 months (IQR 7.5-24.5).

Microscopy and PCR performed on 3 monthly samples collected from birth detected *P. falciparum* infections at least once at three monthly visits in 33% of cases and 15% of controls. This finding predicted, compared to only negative visits, an increased risk of admission with severe malaria (OR 3.70, 95% CI 1.25–10.92, $p = 0.018$) and particularly if a multiclonal infection was ever detected (OR 15.32, 95% CI 1.49 –157.40 $p = 0.022$). Parasite positivity in the most recent sample before admission gave a similar odds ratio as parasite positivity at any time (OR 3.62, 95% CI 0.84–15.71, $p = 0.085$). Assessment of the risk of severe malaria with regards to parasite status at different ages, number of parasite positive visits, or to cumulative number of clones was restricted by limited data for subgroup analysis. Since samples for parasite genotyping were available only from three-monthly visits, we further assessed the prevalence of antibodies against *P. falciparum* schizont extract as a marker of exposure. No

association was found between detection of these antibodies and overall risk of severe malaria (OR 0.76, 95% CI 0.30–1.89).

Children who developed cerebral severe malaria appeared to be less exposed prior to the admission compared to children who developed other syndromes of severe malaria. Cerebral malaria cases never had more than one clone detected prior to admission and the proportion with antibodies to schizont extract were 59% compared to 86% of children with other syndromes. Moreover, the odds of developing cerebral malaria was lower if antibodies were ever detected (OR 0.15, 95% CI 0.03–1.23)

4.2 STUDY II:

High-speed shaking of frozen blood clots for extraction of human and malaria parasite DNA

Blood clots remaining after serum separation are often stored and frozen without anticoagulant, and the sample type can be challenging to dissolve for pipetting and DNA extraction. This study aimed to improve methodology for preparation of frozen blood clots for DNA extraction for PCR templates in study I. Three methods for clot dispersion were evaluated for their ability to disrupt frozen residual blood clots in preparation for extraction of DNA for PCR templates. The chemical protocol (Puregene, Qiagen) failed to disperse the blood clot, only the outer part of the clot was dissolved, with a large core remaining unaffected. Centrifugation of the clot through a plastic sieve (Clotspin® Basket, Qiagen) disrupted parts of the clots; however, large clot particles remained stuck in the sieve. High speed shaking succeeded in efficiently dispersing the clots. After shaking the original tube for 40 seconds, the blood clots were totally

homogenized in the lysis buffer. The product was easily pipetted, consisting of mainly dissolved material. Degree of dispersion was correlated to the success of PCR detection of *P. falciparum* parasites. DNA yield was also highest in the samples processed by high-speed shaking.

The performance of the high-speed shaking method was then further tested on field samples. Genotyping of the human thalassemia gene was performed by PCR in 316 blood clot samples processed by high speed shaking prior to DNA extraction. The success rate of amplification after shaking was 92%, compared to 27% with the vortexing method previously used. Moreover, PCR for detection of *P. falciparum* parasites was performed on clot samples extracted by the high-speed shaking protocol, generating a parasite prevalence of 8.8% compared to microscopy 4.3%. This level of detection demonstrates the high quality of the isolated PCR template.

4.3 STUDY III:

Asymptomatic multiclonal *Plasmodium falciparum* infections carried through the dry season predict protection against clinical malaria in the following high transmission season

The genetic diversity of parasites carried through the dry season on risk of clinical malaria was assessed in a longitudinally followed cohort with 225 individuals aged 2-25 years resident in Kambila, Mali, where malaria transmission is intense during a sharply demarcated rainy season.

A total of 1064 samples (mean 4.72 samples per individual) were analysed by *msp2* PCR. Parasite prevalence by PCR was 14.2%, 15.1%, 34.7%, 30.7% and 16.4%, at the respective five time points (May 2006 after the 6 month dry season, July 2006, October 2006, December 2006 and April 2007). The youngest

children aged 2-4 years had the lowest prevalence both by microscopy and PCR and the highest mean parasite densities at cross-sectional surveys. The genetic diversity was extensive with 104 *m*sp2 alleles detected in 249 PCR positive samples, being highest in children 5-10 years old and increased during the peak of transmission in all age groups.

A total of 298 episodes were recorded during the study follow-up, 0-5 episodes per individual in children and 0-1 in adults. Among children 79.0% experienced at least one malaria attack compared to 8.2% of adults. Detection of multiclonal infections before the rainy season conferred a decreased risk of malaria, compared to being parasite negative, when assessing time to first malaria episode (baseline May 2006 HR 0.28, 95% CI 0.11-0.69 and baseline July 2006 HR 0.40 95% CI 0.18-0.87, adjusting for age and previous treatment) also when recurrent events of clinical malaria was included in the risk assessment. Carriage of one clone through the dry season did not affect the risk in the subsequent malaria transmission season.

4.4 STUDY IV:

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

To assess longevity of immune responses to malaria after a single exposure we compared levels of malaria-specific antibody and proportion of MBC specific for three different malaria antigens between 47 travelers who returned to Sweden with *P. falciparum* malaria, 8 previously unexposed individuals and 14 adults living in an area of high malaria transmission. Time from malaria admission until the cross-sectional sampling for this study ranged between 1 and 16 years.

P. falciparum-lysate-specific antibody levels were above naive control levels in 30% of the travelers, whereas the level of AMA-1, MSP-1₄₂, and MSP-3-specific Ab levels were similar to the unexposed population. In contrast, 78% of travelers had MBCs secreting IgG specific for at least one malaria antigen (59, 45, and 28% for AMA1, MSP-1₄₂, and MSP-3, respectively) suggesting that malaria-specific MBCs are maintained for longer than the cognate serum Abs in the absence of re-exposure to parasites. Five travelers maintained malaria antigen-specific MBC responses for up to 16 years after successful treatment of malaria (and had not traveled to malaria-endemic regions in the intervening time). There was no difference in the relative proportions of atypical MBC, activated B-cells, classical MBC, plasma cells or immature B-cells between travelers and malaria-naïve adults. No correlation with time from malaria admission and frequency of malaria-specific MBC was found.

5 DISCUSSION

The studies presented in this thesis contribute to the understanding of how immune responses are acquired in relation to exposure to the polymorphic *P. falciparum* parasite. More specifically, they investigated how the genetic diversity of the *P. falciparum* parasites encountered during childhood relate to the risk of malaria (uncomplicated and severe), as well as whether maintenance of immune memory against *P. falciparum* antigens is maintained without repeated re-exposure.

This thesis demonstrates that a single encounter with the malaria parasite can induce immunological memory to *P. falciparum* antigens that lasts for many years, however asymptomatic multiclonal infections carried through the dry season were associated with protection against malaria suggesting that the presence of parasites contributes to clinical protection at re-exposure. In the youngest age group repeated exposure to the parasite was not associated with protection against severe malaria. This variety of findings represents the multifaceted concept of protective immunity to malaria, and also that mechanisms of protection may vary depending on age.

Extensive epidemiological data shows that immunity to severe malaria develops early in life in exposed individuals and it has been proposed that for non-cerebral severe malaria, this protection is achieved after only one-two infective bites (Gupta *et al* 1999). Severe malaria anemia primarily occurs in young children, and postponing the exposure to older age reduced the incidence of this severe malaria syndrome (Aponte *et al* 2007). In study I children who developed severe malaria were more exposed compared to matched controls, and especially children who developed non-cerebral severe malaria syndromes. The finding of

up to 6 different alleles detected prior to the severe malaria admission argues against the notion that children who succumb to severe forms of the disease are previously naïve to malaria. For children with severe malaria with impaired consciousness the prevalence of antibodies (as a marker of exposure) from birth up to the time of admission was lower compared to children with other syndromes and no child had more than one clone detected prior to the severe malaria admission, indicating a lower degree of exposure and force of infection. Although the study provided new insights about parasite exposure preceding a severe malaria episode, the question of how many infections that are needed to achieve protection from severe disease remains to be answered. Moreover, further mechanistic studies could elucidate whether immunity to severe malaria differs from that to mild disease or if this immunity is only dependent on the number of encountered infections.

Study III of this thesis demonstrated that individuals with multiclonal infections detected before the transmission season, presumably carried over several months through the dry season, were at reduced risk of malaria during transmission season. The finding of humans living in malaria endemic areas that are chronically infected with parasites and simultaneously protected from symptoms of malaria was initially described by Sergent and Parrott and termed “premunition” (Sergent and Parrott 1935). More recent and detailed studies of asymptomatic carriers have reported both protection from clinical malaria in children with multiclonal infections (Bereczky *et al* 2007, Färnert *et al* 1999, Al-Yaman *et al* 1997) and increased risk of subsequent clinical malaria associated with multiple clones (Branch *et al* 2001, Henning *et al* 2004, Felger *et al* 1999). These seemingly contradictory results might be explained by several factors: (i) the different age groups studied with a tendency for increased risk at younger age groups, (ii) transmission intensity, since the ability to harbor multiclonal

infections is different depending on transmission level (Färnert *et al* 2009) (iii) difference in the duration of an infection, i.e. having a set of parasite clones continuously present is beneficial to the host and leading to a state of premunition whereas recent inoculations and increases in the number of clones might rather be a determinant of risk.

Several studies that report an association with protection from clinical malaria and carriage of multiple clones do however not report the same effect of carriage of single clones. In a majority of studies sample size has restricted more detailed assessment of the effect of higher number of clones (>2 per individual). Presumably carriage of one clone in an asymptomatic individual would not be dramatically different from carriage of e.g. two clones, so the fact that the lack of association with protection has been consistently reported is intriguing.

Furthermore, clearance of infections by repeated full courses of antimalarials during six months within a trial of intermittent preventive treatment did not markedly affect the diversity of *P. falciparum* infections (Liljander *et al* 2010). Nonetheless, multiclonal infections predicted a reduced risk of malaria only in the placebo group, thus suggesting that it is not only the number of clones present at a single time point, but infections over a preceding period that influence malaria immunity. These findings highlights the advantage of careful interpretation of results from molecular epidemiology studies, and the benefit of follow-up with sampling from multiple time points so that dynamics and duration of infections can, together with age and transmission intensity, be considered in the analysis.

The association between multiclonal infections and a reduced risk of clinical malaria has been reported in several studies. The finding merits further understanding of mechanisms underlying this protection. Since antibody

responses often are short-lived, one hypothesis is that the constant presence of parasites stimulates and maintains a broad antibody response, which could explain the results in study III. This is also supported by the recent findings from Tanzania where children with multiclonal infections have broader antibody responses and that in combination they predicted a higher level of protection than they did individually (Rono *et al* 2013).

The use of capillary electrophoresis in a DNA sequencer for fragment analysis, generates reproducible high resolution sizing at base pair precision, and enables more detailed assessments than gel based methods used for *m*sp2 genotyping (Liljander *et al* 2009). Here, most alleles were found at low frequencies, however, certain alleles were repeatedly detected in both Mali and Kenya. For instance, the FC291, FC329, IC471 and IC509 alleles were found in several individuals at both sites. These alleles have also been reported in other studies using the same genotyping technique, both in mild and severe infections in Uganda (Kiwuwa *et al* 2013). Identical fragment lengths might represent identical sequences, but sequencing would be required to confirm this possibility. The finding of specific *m*sp2 alleles that appear frequently in both asymptomatic and disease causing infections could be suggestive of a survival advantage for the parasite and warrants investigation.

Parasite prevalence and number of clones is generally likely to be underestimated, especially if transmission is low and/or samples come from asymptomatic individuals. Moreover, sampling and storage of blood can affect the limit of PCR detection (Färnert *et al* 1999), and the fact that blood was collected only on filter paper in study III might have underestimated the diversity of parasite populations. In study I, the available material from the large cohort study was residual blood clots saved after serum preparation. To increase de

DNA yield we developed a new method for clot dispersion with high speed shaking. The parasite density as well as number of clones can fluctuate within an individual, with clones sometimes being sequestered in tissues or present at low levels not detectable by PCR (Färnert *et al* 1997, Delley *et al* 2000). This was demonstrated in a study in asymptomatic Tanzanian children who showed a non-random 48-hour periodicity in the appearance of individual parasite clones (Färnert *et al* 1997) and it has been suggested that cross-sectional molecular epidemiology studies should be performed with double sampling with a 24-hour interval (Färnert 2008, Mueller *et al* 2012). Long sampling intervals in longitudinal studies are also likely to contribute to underestimation of number of infections, such as in study I where three-monthly sampling was performed. Analysis of antibodies against parasite schizont extract however provided additional information about previous parasite exposure.

With decreasing transmission in many areas of the world, naturally acquired malaria immunity will also decline, and sustained efforts become important to avoid outbreaks such as in Madagascar in the 1980's (Deloron *et al* 1992). In study IV, we demonstrated the presence of long-lived malaria specific memory B-cells for up to 16 years in the absence of malaria exposure. This result is in agreement with studies from areas of low transmission (Wipasa *et al* 2010) and decreasing transmission (Ndungu *et al* 2012), yet the Swedish population provided even stronger evidence that MBCs are maintained also in absence of exposure. The association of these long-lasting MBC's and protection from clinical malaria needs to be studied further, as well the decay kinetics of antibodies and MBCs and other factors contributing to the maintenance of immune responses.

A causal relationship between expanded compartments of atypical MBC in repeatedly malaria exposed individuals has not been established, although observations point towards such a connection (Weiss *et al* 2011, Illingworth *et al* 2013). Atypical MBCs were not found to a greater extent in Swedish travelers (study IV) compared to healthy controls, suggesting that continuous exposure to the parasite drives the expansion of these cells, and possibly more specifically the asymptomatic infections, since a trend towards higher proportion of atypical MBCs were found in individuals who were asymptotically infected through the dry season (Weiss *et al* 2009).

In summary, the studies presented in this thesis have contributed new knowledge on how parasite exposure relates to malaria risk and malaria-specific immune responses. Children who develop severe malaria are more exposed early in life compared to community matched controls, a finding that argues against the idea that severe malaria develops only after the first encounters with the malaria parasite. A novel method for the preparation of frozen residual blood clots remaining after serum separation and stored without anticoagulant has been developed and can be used by researchers in any field of science that requires extracted DNA. The importance of asymptomatic multiclonal infections for protective immunity in children has been confirmed in a setting of seasonal malaria. Finally, for the first time demonstrated in a population residing in a malaria-free environment, malaria-specific MBC are induced in a majority of patients after a natural *P.falciparum* infection and are maintained for up to 16 years (or longer).

6 CONCLUSIONS AND FUTURE DIRECTIONS

- ❖ Children who develop severe malaria are more exposed to *P. falciparum* infections from birth compared to matched controls, although to a lesser extent with regards to cerebral malaria compared to other syndromes
- ❖ High-speed shaking is an efficient way of disrupting frozen residual blood clots for the purpose of DNA extraction
- ❖ Multiclonal infections carried through the dry season predict a reduced risk of febrile malaria in the ensuing transmission season
- ❖ A single *P. falciparum* infection can induce malaria-specific MBCs that are maintained for up to 16 years (or more) in individuals who live free of malaria

Asymptomatic multiclonal infections have repeatedly been associated with protection from febrile malaria in children but not infants. Elucidating the details of underlying immune mechanisms that confer this protection will provide important insights about naturally acquired immunity.

To systematically assess the effect of carriage of multiclonal infections over time, a longitudinal cohort study where a proportion of individuals were given anti-malarial treatment to clear parasites, would further elucidate the importance of the presence of the parasites *per se* for subsequent protection against clinical malaria.

Malaria-specific MBCs have been demonstrated to be long-lived. Future projects should aim for a more detailed study of MBC specificities and their relation to protective immunity, preferably with high-throughput methods. Aspects of interplay between asymptomatic infections and the generation of immunological memory also warrant investigation.

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