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SURFACE ANTIGENS AND VIRULENCE IN *PLASMODIUM FALCIPARUM* MALARIA

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To my dear dear boys

ABSTRACT

Plasmodium falciparum is an intracellular protozoan that may cause severe forms of malaria. It is a major world health hazard and reaps the highest toll among the children and pregnant mothers of the developing world. An *Anopheles* mosquito vector injects the pathogen when taking a blood meal. After multiplication in cells of the liver, the parasite escapes and infects red blood cells in a cyclic manner and this is when the clinical manifestations of malaria as a disease become apparent. The parasite causes the infected red blood cells to adhere to each other (rosetting) and to the blood vessel walls (cytoadherence) by exporting highly variable and adhesive PfEMP1 proteins to the erythrocyte surface. Different immunological and genetic properties of the host as well as parasite specific clonal phenotypes determine the outcome of the encounter. We have investigated the parasite side of these events by performing a clinical case-control study where we sampled a score of isolates from patients with severe or mild malaria. These investigations took part in two endemic areas in Uganda; Apac a small rural community in the northern part of the country and Kampala, the capital. The work was partitioned into four different headings where we aimed to explore different aspects of what separates the parasites we found in severe disease patients from the uncomplicated group. Primarily we sought to characterize the sequences of the *var* genes encoding PfEMP1 expressed in the different patient groups. Through implementing a semi-quantitative PCR amplifying cDNA, massive scale sequencing and a bioinformatics pipeline we could identify degenerated amino-acid motifs that were either statistically overrepresented in PfEMP1 sequence tags sampled from patients with severe- or mild malaria. These were put in a structural-functional context through 3D modeling and potential sites for receptor interaction were identified. The expression of the *var* genes in the fresh isolates was further explored in a temporal context to understand the timing of *var* gene transcription in the patient. We compared semi-and absolutely quantified *var* genes in a subset of the Ugandan isolates. We chose an approach where we constructed a quantitative-PCR assay that enumerated the amounts of individual *var* genes in a heterogeneous solution. The transcription patterns were individual to each isolate and we found that dominance of genes could flux between developmental stages. In a separate study, two of the isolates were chosen to be included in a larger genomics survey of the entire genome by use of a 70-mer oligonucleotide micro-array platform. Size fractionated gDNA from a panel of parasites were hybridized under stringent conditions and cross referenced against the 3D7AH1 genome parasite. The assay could identify a number of gene copy number polymorphisms that were associated to proliferative properties of the parasites, drug resistance or putative invasion related genes. The microarray results were confirmed by PCR and fluorescent *in situ* DNA hybridization. Finally we studied the growth of fresh *in vitro* adapted field isolates of children from severe- or mild malaria and found a correlation between the level of rosetting and their multiplication rates. By disrupting rosetting with either anti-immunoglobulin antibodies, heparan sulfate or antibodies to PfEMP1 we could also block the growth of the parasite facilitated by PfEMP1 rosetting. Taken together, these findings argue that *P. falciparum* specific pheno- and genotypes exist that may predispose for the development of severe malaria. In conclusion, we have found specific molecular evidence for inter-parasite differences in *P. falciparum* that in the future may be exploited in intervention strategies.

LIST OF PUBLICATIONS

This thesis is based on the following papers, which will be referred to by their roman numerals.

I. **Normark J**, Nilsson D, Ribacke U, Winter G, Moll K, Wheelock CE, Bayarugaba J, Kironde F, Egwang TG, Chen Q, Andersson B, Wahlgren M.
PfEMP1-DBL1alpha amino acid motifs in severe disease states of *Plasmodium falciparum* malaria.
Proc Natl Acad Sci U S A. 2007 Oct 2;104(40):15835-40.

II. Ribacke U, Mok BW, Wirta V, **Normark J**, Lundeberg J, Kironde F, Egwang TG, Nilsson P, Wahlgren M.Genome wide gene amplifications and deletions in Plasmodium falciparum.
Mol Biochem Parasitol 2007 Sep;155(1):33-44.

III. Blomqvist K, *__Normark J__*, Nilsson D, Ribacke U, Orikiriza J, Trillkott P, Byarugaba J, Egwang TG, Kironde K, Andersson B, Wahlgren M Developmental Shift in *var* gene Transcription in Fresh *Plasmodium falciparum* isolates.
Manuscript

IV. Ribacke U, Moll K, **Normark J**, Vogt AM, Chen Q, Flaberg E, Szekely L, Hultenby K, Egwang TG, Wahlgren M
Merozoite invasion in *Plasmodium falciparum* malaria is facilitated by PfEMP1 mediated rosetting.
Manuscript

* These authors contributed equally to this work

CONTENTS

LIST OF ABBREVIATIONS

AMA-1	Apical Membrane Antigen-1
ARDS	Acute Respiratory Distress Syndrome
ATS	Acidic Terminal Segment
CGH	Comparative Genomic Hybridizations
CIDR	Cysteine rich Interdomain Region
CM	Cerebral Malaria
CR1	Complement Receptor-1
CSA	Chondroitin Sulfate A
DBL	Duffy Binding Like protein or domain
DIC	Disseminated Intravascular Coagulation
DRM	Detergent-Resistant Membrane
EGF	Epidermal-growth-factor
EPO	Erythropoetin
GAG	Glycosaminoglycans
GPI	Glycosylphosphatidylinositol anchored proteins
HS	Heparan Sulfate
ICAM-1	Intercellular Adhesion Molecule-1
IE	Infected Erythrocytes
KAHRP	Knob-Associated Histidine-Rich Protein
MESA	Mature parasite-infected Erythrocyte Surface Antigen
MSP	Merozoite Surface Protein
NTS	N-terminal Segment
PAM	Pregnancy Associated Malaria
PEXEL	*Plasmodium* Export Element
PfEMP1 *(var)*	P. falciparum Erythrocyte Membrane Protein 1
PfEMP3	*P. falciparum* Erythrocyte Membrane Protein 3
PfRh	Reticulocyte binding protein
PfSUB1	*P. falciparum* Subtilisine like serine protease-1
PMNS	Post-Malaria Neurological Syndrome
PVM	Parasitophorous Vacuolar Membrane
RESA	Ring infected Erythrocyte Surface Antigen
RIFIN (*rif)*	Repetitive Interspersed protein *(gene encoding* RIFIN*)*
SERA	Serine Repeat Antigen
STEVOR *(stevor)*	Sub-Telomeric Variable Open Reading Frame protein *(gene encoding* STEVOR*)*
SURFIN *(surf)*	Surface associated Interspaced protein *(gene encoding* SURFIN*)*
TNF	Tumor Necrosis Factor
TRAP	Trombospondin Related Apical Protein
TSP	Thrombospondin
var	Gene encoding P. falciparum Erythrocyte Membrane Protein 1
VCAM-1	Vascular Cell Adhesion Molecule-1
VSA	Variable Surface Antigen
VTS	Vacuolar Transport Signal

1 INTRODUCTION

Malaria has plagued humanity since the dawn of mankind. It is a vicious killer of mainly children and pregnant women, and as with many scourges afflicting humanity, the yoke falls most heavily on the poor. The overall death toll caused by malaria has varied little since the mid 1950´s when mass eradication of the anopheles mosquito vector and antimalarial prophylaxis were initiated. It is a major contributing factor of underdevelopment and paucity in tropical and subtropical regions of the world. Recently, however some progress has been made in controlling the disease. The *World Malaria Report 2008* (http://www.who.int/malaria/wmr2008/) found an estimated 247 million cases of malaria and 881 000 deaths from the disease, mostly among children in Africa, meaning that it remains one of the world's leading causes of death. Nevertheless, several countries had achieved a sharp fall in the number of people affected by malaria after increasing control measures. These areas had achieved high coverage of measures to control malaria, including greater use of bed nets treated with insecticide, better provision of antimalarial drugs, and spraying insecticide inside houses. This may be an effect of the recent surge in economic growth in Asia, but can also be attributed to a more concerted effort of the world community in dealing with neglected diseases. Facts remain, that malaria is a disease that can be cured, inexpensively and can be prevented with relatively small means.

PLASMODIUM AND APICOMPLEXAN PROTOZOA

The Apicomplexan phylum consists of a wide spectrum of eukaryotic organisms. It includes protozoan parasites of both veterinary and human importance such as *Toxoplasma spp*, *Theileria spp* , *Eimeria spp, Cryptosporidium spp* and *Babesia spp.* All these parasites have complex lifecycles and can either utilize invertebrate vectors or pass directly between vertebrate hosts. Some, including the *Plasmodium spp,* utilize arthropods for their transmission. All apicomplexans share the common feature of having an apical complex, hence the name. This structure is central to invasion of host cells and to the pathogenesis of the parasites.

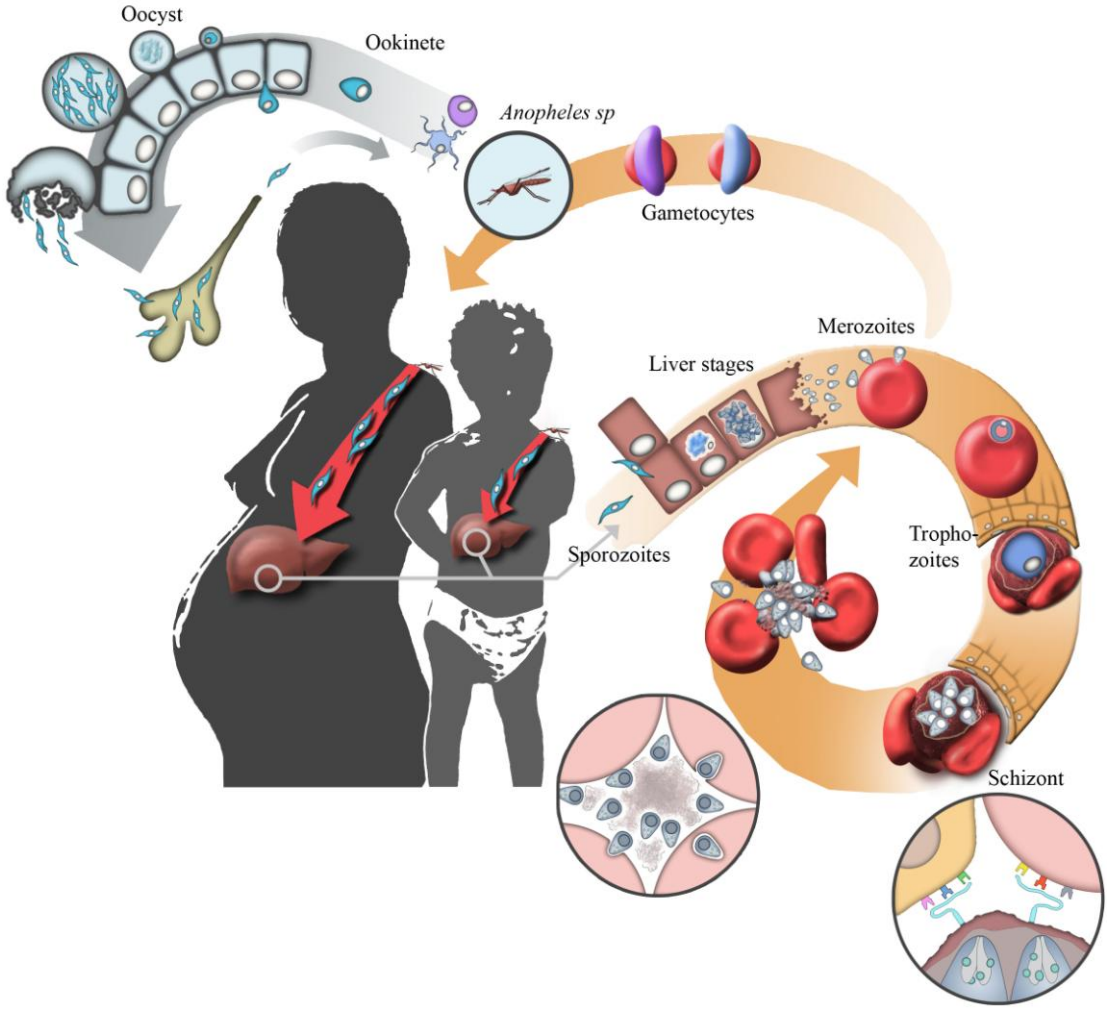

Figure 1. The *Plasmodium falciparum* life-cycle

1.1.1 Lifecycle

Plasmodium spp. infect the mammalian host by female *Anopheles* mosquitoes injecting sporozoite forms into the host circulation when taking a blood meal. Ejection occurs during the first probing phase of the mosquito bite and the numbers delivered in each bite rise with the parasite density in the salivary glands of the insect (Frischknecht et al., 2004). The sporozoite, a spindle shaped extracellular form migrates first through the skin (around 30 min) (Vanderberg et al., 2004) and then rapidly with the circulation to the liver. There it actively invades hepatocytes after traversing the Kuppfer cell lining of the sinusoids, through the space of Disse (Danforth et al., 1980; Pradel et al., 2001). This was recently documented in vivo by (Frevert et al., 2005) in a murine model. Each invading sporozoite differentiates and divides mitotically into thousands of merozoites. Transcriptional studies of liver stage parasites suggest that merozoites formed in the hepatocytes are similar in structure and function to those that are subsequently formed in the erythrocytes (Wang et al., 2004). Merozoites share characteristics of other apicomplexan infective forms such as the tachyzoites of *T. gondi* in that they include a polarized morphology and carry apical organelles that secrete their contents during host-cell invasion. Evidence suggest that merozoites are released from infected

hepatocytes as merosomes, packets of hundreds of parasites surrounded by host cell membrane (Sturm et al., 2006; Tarun et al., 2006; Baer et al., 2007). These studies were performed in *P. berghei* murine models and have yet to be investigated in the human setting. As the merozoites are ejected into the circulation they invade erythroytes. This is a complicated and dynamic multistep process which initiates the phase of the infection that encompasses all the clinical manifestations of the disease. *Plasmodium spp* spend 24-72 hours growing in the erythrocyte, developing from a ring stage trophozoite, successively to mature trophozoites and after subsequent mitotic nuclear division into schizonts. A critical step in plasmodium survival and growth in the erythrocyte is the formation of a parasitophorous vacuolar membrane. It is established during invasion and surrounds the parasite during intracellular growth and manifests complex structural and transport functions at different intraerythrocytic stages (Aikawa et al., 1978; Atkinson et al., 1990; Bannister et al., 1990). After segmentation (schizogony) is complete and new merozoites are formed the erythrocyte bursts. This process involves a timed release of proteases stored in the apical secretory organelles detailed in Yeoh 2007. The merozoites are released into the circulation, free to invade new erythrocytes. The cycle is re-enacted with exponential growth as a result and is arrested either when the host succumbs to the disease or the parasite is controlled by an effective immune response or chemotherapy. The plasmodium life cycle continues through a developmental switch either during schizogony (Bruce et al., 1990) or after merozoite invasion, within 16 hours of the lifecycle (Mons 1986) into sexual gametocyte forms. A vast literature exists on various factors reported to induce gametocyte production (Alano et al., 1990; Talman et al., 2004). The present knowledge, however suggests that there are no specific molecular or cell- mediated mechanisms, other than a general parasite response to adverse growth conditions linked to gametocytogenesis. There is evidence pointing towards that ineffective treatment and increased rates of recrudescence are associated to increased gametocyte production (Price et al., 1999). The gametocytes can remain in the circulation for ten days or more. The details of how the sexual forms survive in the circulation for such a prolonged period of time are still not clearly understood. Work by (Crabb et al., 1997) and (Day et al., 1998) indicate that gametocytes utilize the same mode of immune evasion utilized by the asexual intraerythrocytic forms that will be detailed further in this thesis. The feeding mosquito, which also is the definitive host, will once more ingest plasmodium infected blood. The male gametocyte divides into eight flagellated microgametes, which escape from the enclosing red blood cell. One microgamete fertilizes the female macrogamete, and the resultant motile ookinete migrates through the cells of the stomach wall. Upon reaching the outside of the mosquito stomach the ookinete encysts, creating a wart-like structure known as an oocyst. In this stage the parasite undergoes asexual clonal expansion resulting in thousands of sporozoites. After rupture of the oocyst the sporozoites migrate to the salivary glands of the mosquito and upon arrival the sporozoites become infective, as the sporozoites in the oocyst are both physiologically and biochemically distinct from those in the glands (Vanderberg 1975; Touray et al., 1992). When the female mosquito feeds, active sporozoites are again injected into the next host and the transmission cycle is complete.

1.2 DISEASE CHARACTERISTICS

The plasmodium genus infective to man can be divided up into five species. These demonstrate different disease characteristics where *P. ovale, P malariae* and to some extent *P. vivax* give rise to a benign, usually self limiting condition. *P. falciparum* causes the feared subtertian malaria, the variety which is attributable to almost all severe symptoms and mortality of the disease. Recent evidence describes *P. knowlesi* as the fifth human malaria species (Cox-Singh et al., 2008; Ng et al., 2008; Tan et al., 2008). According to archival data it has often been misdiagnosed as *P. malariae*. The nature of the clinical disease depends very much upon the pattern and intensity of malaria transmission in the geographical location, which determines the degree of protective immunity which in turn affects the clinical profile.

1.2.1 General features

Infections with all the five different malaria species have many clinical features in common. These are related to the liberation of fever-producing substances, especially during schizogony, and the fact that every infected red blood cell will be destroyed within 48-72 hours post infection. Most patients also report headache, lassitude, diarrhea, muscular discomfort and malaise, either singularly or in combination. The common features are:

i. Fever, often irregular in *P. falciparum*. The fever is believed to be caused by outpouring of pro-inflammatory cytokines into the host circulation in response to erythrocyte destruction products and parasite derived pyrogens. The tumor necrosis factor (TNF) has been the most intensely studied in this regard (Scuderi et al., 1986; Kern et al., 1989; Molyneux et al., 1991). Studies show that GPI anchors derived from parasite membrane material induce high levels of TNF secretion. In *in vivo* experiments in humans TNF secretion was neutralized by the injection of murine monoclonal antibodies which also abolished fever (Kwiatkowski et al., 1993). The pattern of regularly periodic fever often does not occur until the illness has continued for a week or more. It depends on synchronized schizogony which is a feature that is often reported in classical malaria.

ii. Haemolytic anemia. The rapid drop in haemoglobin during acute infection and the slower decline in chronic infection appear to be due to increased extravascular haemolysis of RBCs with a concomitant failure of the bone marrow to increase red cell production to compensate for these losses (Jakeman et al., 1999; Lamikanra et al., 2007).

iii. Splenomegaly. The spleen enlarges in the acute attack in all sorts of malaria. With repeated infections the spleen may grow to massive size and result in a secondary hypersplenism.

iv. Jaundice. A mild jaundice may due to haemolysis appear in all types of malaria, however jaundice due to liver involvement is a phenomenon only seen in *P. falciparum* infections.

1.2.2 P. falciparum

The important difference between *P. falciparum* and the other human plasmodia is the capacity to cause severe disease, with the exception of *P.knowlesi* which has been reported to cause cerebral malaria. The special aspects of *P. falciparum* includes the high asexual multiplication potential, the ability to invade erythrocytes of all ages as well as the efficient evasion of host immunity due to sequestration in the peripheral circulation and antigenic variation. Nearly all of the roughly million of deaths that occur each year can be attributed to *P. falciparum* infection. Following a single exposure, the patient will either die in the acute attack or survive with residual anemia and have attained some limited immunity against the disease. The initial symptoms of *P. falciparum* are nonspecific and similar to the limited mild febrile illness common to all malaria species as described above. If ineffective drugs are given, the patient lacks the capacity to mount an effective immune response or if the treatment is delayed the parasite burden continues to increase and severe malaria may ensue. A patient may progress from having minor symptoms to having severe disease within a few hours. This usually manifests with one or more of the following: unrousable coma (cerebral malaria); severe anaemia; renal failure; pulmonary oedema or acute respiratory distress syndrome (ARDS); hypoglycaemia; circulatory collapse or shock; spontaneous bleeding from gum, nose, gastrointestinal tract, *etc.* and/or substantial laboratory evidence of disseminated intravascular coagulation (DIC); repeated generalized convulsions; acidaemia or acidosis including hyperlactataemia. By this stage, mortality in people receiving treatment has risen to 15-20%. If untreated, severe malaria is almost always fatal. *P. falciparum* also gives rise to pregnancy associated malaria, which is associated with both maternal and infant complications.

1.3 SEVERE MALARIA

There are considerable differences in the manifestations of severe malaria between adults and children. While severe anemia is the most common manifestation in very young children, cerebral malaria is the predominant cause of death in older children and adults (Dondorp et al., 2008b). Interestingly, in areas of higher malaria transmission children, on average, encounter malaria at a younger age and the mean age of clinical cases is lower. Malarial anaemia tends therefore to be relatively more important under high transmission settings and cerebral malaria tends to gain in importance under lower transmission settings (Snow et al., 1997). This is thought to reflect the immunological development of the patient and the relationships are not well understood. There is also evidence that the overall parasite density in patients suffering from severe malaria is higher than in patients presenting mild symptoms (Dondorp et al., 2005). In this study, the biomass of parasites in the circulation was calculated through quantification of PfHRP2 and here the biomass of parasites was also found to be higher in the patients that died. The pathogenic mechanisms that lead to severe malaria has been mapped in greater detail in cerebral malaria, severe anemia and to some extent the ARDS. Furthermore, placental malaria is now well characterized. As such these conditions will be reviewed as follows.

1.3.1 Cerebral malaria

Cerebral malaria (CM) is defined as unrousable coma (non-purposeful response or no response to a painful stimulus ie. scoring less than 3 on the modified Blantyre coma scale (Molyneux et al., 1989) in falciparum malaria. The onset of coma may be gradual after an initial stage of confusion or may be abrupt after seizures. The unconsciousness f the post-ictal state is usually short and persistence of unconsciousness beyond 30 minutes after convulsions should be considered as CM. Mortality is significantly higher in patients of cerebral malaria when associated with other complications such as acute renal failure, acute respiratory distress syndrome, jaundice, *etc.* Hypoglycaemia resulting from malaria or quinine treatment can also cause coma and, if it is severe and protracted, may result in death or permanent brain damage (White et al., 1987). Common to all pathologic descriptions of CM is the presence of large numbers of parasitized red blood cells in the microvasculature (Marchiafava et al., 1894; Spitz 1946; Pongponratn et al., 1991); however, high parasitemia in the peripheral circulation does not necessarily correlate to cerebral malaria in relation to non cerebral severe disease manifestations (Pongponratn et al., 2003) although the overall burden of parasites is higher in those with severe disease (Dondorp et al., 2005). The study of Pongponratn and coworkers (1991) also gives support to the fact that all patients who develop coma show significant sequestration of infected erythrocytes (IE) in the brain microvasculature. Thus the prevalent theory on the pathogenesis of the disease has been that that sequestration of IE in the brain leads to mechanical impairment of vascular perfusion, and in turn to cerebral anoxia and coma. As is the case for many diseases affecting the brain, far more is known about the pathogenesis of murine CM than human CM. The most obvious reason for this is that sizeable numbers of murine brains can be studied at different phases of the illness, but human brain studies of CM are limited to those done at autopsy. Throughout the past two decades there has been increasing evidence that a number of immune-related mechanisms first noted in murine CM, such as TNFα production (Grau et al., 1987) blood-brain barrier breakdown and endothelial cell damage, are also involved in the pathogenesis of human CM (Grau et al., 1989; Neill et al., 1993; Brown et al., 1999; Brown et al., 2001; Combes et al., 2004). Human and murine data also suggest a potential role for other immunological or inflammatory factors in CM pathogenesis, including vascular cell adhesion molecule-1 (VCAM-1), intracellular adhesion molecule-1 (ICAM-1) (Turner et al., 1994; Chakravorty et al., 2005), heme oxygenase-1 (Schluesener et al., 2001), nitric oxide (Senaldi et al., 1992), kynurenic acid metabolites (Sanni et al., 1998), and interferon gamma (Grau et al., 1989). However, it should be kept in mind that the murine parasites do not, sequester prominently and do not express the *P. falciparum* adhesin PfEMP1 that mediates the binding of the IE. Current models of human CM postulate a contribution of multiple factors, including microvascular sequestration and blockage leading to local ischemia; cytopathic hypoxia, in which oxygen supply is adequate but cellular use of oxygen is not; and up-regulation of numerous immune or immune-related responses, all of which combine to lead to blood-brain-barrier breakdown, microglial and astrocyte activation, and damage or death of microglia, astrocytes, and neurons (Hunt et al., 2006). CM is fatal in 15–20% of cases (in optimal settings) and residual neurological sequele is seen in a further 10% of children and 1–3% of adults

(Warrell et al., 1982; Brewster et al., 1990). These have been described as the post-malaria neurological syndrome (PMNS) which includes a variety of neurological deficits. PMNS is usually transient, but in some cases permanent disability remains (Senanayake et al., 1994). Lately interest has been focused on more subtle neurological sequaele since CM has been associated with long term cognitive impairment in one out of four child survivors (John et al., 2008).

1.3.2 Severe anemia

Anemia of varying degree is a common accompaniment in severe malaria. Severe anemia is scientifically defined as haemoglobin lower than 5g/dl or haematocrit lower than 15%. The increased clearance of infected cells is readily explained by the rupture of cells after completion of the parasite's intra-erythrocytic life cycle and opsonisation and clearance of intact infected RBCs. Rather less obvious is why and how uninfected cells are also cleared. It has been estimated that approximately 10 uninfected cells are cleared from the circulation for every infected cell and so the clearance of uninfected cells is of crucial importance for the development of malarial anemia (Jakeman et al., 1999). This could in part be due to failed invasion of merozoites (Layez et al., 2005),or as suggested previously and by recent data from (Evans et al., 2006) who show in a rodent model that the principal cause of malarial anemia is caused by uptake of uninfected erythrocytes by monocytes and macrophages. Increased deformability of uninfected red blood cells predisposes them for clearance and also contributes to severe anemia in humans (Dondorp et al., 2003). Marrow hypoplasia and subsequent dyserythropoesis in acute as well as more chronic stages of malaria are features attributed to suppression of the normal response of erythropoetin (EPO) (Phillips et al., 1992). EPO synthethase suppression by cytokines such as IFNγ and TNFα, which are upregulated in the bone marrow during *P. falciparum* infection, may as well play a role (Clark et al., 1988; Miura et al., 1991).

1.3.3 Respiratory distress

Pulmonary edema/Acute respiratory distress (ARDS) is a grave complication of severe malaria which entails a high mortality rate. It may develop early after one or two days of treatment or may appear late in the course of the disease when the patient's general condition is improving. Fluid overload may be an important contributory factor, but in some patients respiratory distress may develop even with normal or negative fluid balance. The first indication of impending ARDS is usually an increase in the respiratory rate in the absence of metabolic acidosis and anemia. Although ARDS are rare in the pediatric population (Taylor et al., 2006), respiratory distress accompanying severe metabolic acidosis is common in children and predicts poor outcome. A large study from coastal Kenya (Marsh et al., 1995) contained the observation that severe respiratory distress alone predicted over 3 times the likelihood of a fatal outcome than did coma alone. It has been proposed that weak contractility of the respiratory muscles restricts the ability of children with severe malaria to compensate for their acidosis and that eventually severely weakened muscles, under the urgency to increase pH, cease functioning through exhaustion (Clark et al., 2003). Lung ultrastructural studies from individuals with fatal *P. falciparum*-induced lung injury indicate endothelial cell cytoplasmic swelling and edema in the lung interstitium, with monocytes and IE adherent within the capillaries(Duarte et al., 1985; MacPherson et al., 1985). *P.*

falciparum infected IEs have been shown to promote oxidative stress *in vitro* together with caspase activation leading to apoptosis in human primary lung endothelial cells (Gimenez et al., 2003). A *P. berghei* ANKA model of respiratory distress showed alveolar capillary membrane disruption and a marked increased production of proinflammatory cytokines such as TNF, IFN-γ, IL-10 and IL-6 both locally in the lung circulation and in the periphery (Lovegrove et al., 2008).

1.3.4 Pregnancy associated malaria

A long time observation by physicians has been that there is an increased severity of malaria during pregnancy. This may result in adverse obstetrical outcomes such as premature delivery, intrauterine growth retardation, complications at partus and perinatal mortality. The mother suffers an increased risk of severe malaria (from 2-10 fold) in mesoendemic regions (Brabin 1983) as well as anemia in regions of high endemicity (Menendez et al., 1995). An estimated 300,000 fetal and infant deaths and 1500 maternal deaths each year can be attributed to pregnancy associated malaria (PAM) (Brabin et al., 2004). Primigravidae mothers are at greatest risk of developing PAM, and the prevalence drops sharply with successive pregnancies (McGregor et al., 1983; Watkinson et al., 1983; Steketee et al., 1996). Peripheral parasite prevalence is also higher in the second trimester of pregnancy and decreases with advancing gestational age (Brabin 1983). Recent work indicates that PAM in the mother also increases the risk of malaria infection in the offspring during the 30 first months of life (Schwarz et al., 2008). The increased susceptibility of both mother and developing child to malaria is dependent on adherence and accumulation of parasite-infected erythrocytes within the placenta and a potentially aggressive immune response in the organ (Walter et al., 1982; Ismail et al., 2000). Unlike other sequestration sites, inflammatory cells are a prominent component of infected placentas (Moshi et al., 1995; Ordi et al., 1998; Rogerson et al., 2003). In particular, monocytes are heavily recruited to the infected placenta by unknown processes and it is unclear if they act in a protective or deleterious manner. In certain cases massive influx of monocytes into the intervillus space has been observed and termed intervillositis or placental inflammation (Ordi et al., 1998). PAM is associated to chondroitine sulfate (CSA) binding of parasitized blood cells as well as other receptors in the placental circulation, described further below.

1.4 PATHOGENESIS OF *P. FALCIPARUM* MALARIA

1.4.1 Invasion

The whole process of merozoite invasion can be divided into defined steps that entail a number of distinct morphological and molecular events. The first phase involves merozoite release and identification of a new potential host cell. The second phase consists of attaining the proper orientation in relation to the erythrocyte through reversible merozoite surface coat antigens. This is succeeded by an entry phase where the surface coat is shed and the parasitophorous membrane is established. The final phase is characterized by the initial modification of the host cell milieu and the transformation of the parasite into a feeding, growing trophozoite.

1.4.1.1 Erythrocyte rupture and merozoite egress

Invasion of a new host cell requires the malaria parasite to primarily exit from its infected cell (Trager 1956; Winograd et al., 1999) Real-time microscopic examination has shown that this is a rapid course of events (Wickham et al., 2003; Glushakova et al., 2005). The mechanics of this process involves the disruption of the vacuolar space the parasite occupies, and rupture of the host cell membrane. Egress of *P. falciparum* involves a sudden increase in intracellular pressure accompanied by biochemical changes that destabilize the host cell cytoskeleton. In combination this causes an almost explosive event that scatters the merozoites into the outside environment (Glushakova et al., 2005). It is not completely elucidated exactly how the rise in intracellular pressure and destruction of the cytoskeleton is brought about. Nonetheless, three main lines can be summoned from the accumulated data. The breakdown of the parasitophorous vacuolar membrane (PVM) and host cell membrane are differentially regulated. It would appear that PVM degradation is an E64 sensitive procedure (Salmon et al., 2001; Gelhaus et al., 2005; Soni et al., 2005). Given that E64 is a highly selective cysteine protease inhibitor; this strongly implicates one or more cysteine proteases in PVM rupture and at least one additional distinct activity in host cell membrane rupture. Evidence is mounting towards a two step process where rupture of the internal vacuolar membrane occur separate and just prior to that of the erythrocyte membrane (Wickham et al., 2003). The serine repeat antigen (SERA) family of proteases, located to the parasitophorous vacuole, has been identified as protease candidates (Miller et al., 2002; Hodder et al., 2003). It has been suggested that the SERA family members perform as a dual function proteins (Blackman 2008). A central domain that acts as a soluable protease and plays a role in egress and terminal domain that interact with partner proteins on the merozoite surface which plays a separate role in merozoite viability post egress. A SERA processing system has been found in the subtilisine like serine protease (PfSUB1) which is stored in an unusual set of granule like organelles (merozomes) in the apical complex of the merozoites (Yeoh et al., 2007).

1.4.1.2 Host cell contact

The initial contact between the merozoite and the erythrocyte may occur on any surface of the free parasite. This recognition and primary adherence is relatively long distance, apparently of low affinity and reversible (Bannister et al., 1990). Videomicroscopic and EM observations show that the contact coincides with dynamic heaving oscillations of the red blood cell membrane (Dvorak et al., 1975).The surface coat of merozoites is largely composed of glycosylphosphatidylinositol (GPI) anchored proteins and attached partner proteins (Sanders et al., 2005). There are nine known GPI anchored proteins present on the *P. falciparum* merozoite surface. Many surface proteins also share similarities aside from the GPI anchor such as cysteine rich domains that potentially play a role in adherence. These domains include epidermal-growth-factor-like (EGF) moieties at the C-terminus of four surface proteins and 6-cys modules (present in Pf12 and Pf38). 6-cys proteins can also be found on the surface of *P. falciparum* gametes and sporozoites (van Dijk et al., 2001; Ishino et al., 2006).

MSP-1 is a dominant GPI anchored antigen and the most abundant protein on the surface (Holder et al., 1984; Miller et al., 1993). It is a member of the merozoite surface protein (MSP) family and thought to be essential to parasite survival. It was first suggested to bind to human and primate erythrocytes in vitro (Perkins et al., 1988)

and it has been extensively studied. In these assays the binding capacity was dependent on terminal sialic acid residues present on erythrocyte membrane proteins. Definitive proof is still lacking as to if MSP-1 confers the initial contact between merozoite and red blood cells. MSP-1 has been shown to be processed into four separate parts by the time merozoites are released from infected erythrocytes (David et al., 1984; Holder et al., 1984; Lyon et al., 1986; Holder et al., 1987) and the important EGF containing domain MSP-1_{19}, can be radically altered without affecting invasion (O'Donnell et al., 2000; Drew et al., 2004). In any case, antibodies to the same C-terminal MSP-1_{19} inhibit invasion in vitro (Blackman et al., 1990). As such it has become the chief malaria vaccine candidate. Although the MSP-1_{19} tertiary structure has been solved the function still remains indefinable.

1.4.1.3 Merozoite re-orientation and attachment

Following initial recognition and contact the merozoite juxtaposes the apical end to the erythrocyte membrane and a tight junction will form. The rhythmic oscillations of the erythrocyte membrane initiated on contact seem to facilitate this process. The exact adhesive and contractile forces the merozoite induces are not known. In contrast to the merozoite surface proteins there is clear evidence that a number of proteins stored in the apical vesicular organelles bind to specific receptors on the red cell membranes. These are directly involved in invasion. A protein thought to be essential in establishing the apical interaction is the apical membrane antigen-1 (AMA-1). It cannot be disrupted through reverse genomics (Triglia et al., 2000) and AMA-1 antibodies specifically inhibit invasion although they do not interfere with initial attachment (Dutta et al., 2003; Healer et al., 2004; Mitchell et al., 2004). AMA-1 has been shown to be stored in the micronemes from the time the organelles are transported to the from the Golgi cisterni along microtubules to the apical prominence of the merozoite. There it is released to the merozoite surface during invasion (Healer et al., 2002; Bannister et al., 2003). It likely represents a link between the weak initial contact involving MSP proteins and irreversible tight relations formed with microneme proteins (Cowman et al., 2006). Two protein families, the Duffy binding like proteins (DBL) (including EBA-175, EBA-140 and EBA-181) and the reticulocyte binding protein (PfRh) family do show promise as candidates for mediating such interactions. DBL proteins bind red blood cells in a sialic acid dependent manner where binding can be abolished through neuraminidase treatment. The receptor binding domains have been defined within the first cysteine-rich region for several of these DBL members (Chitnis et al., 1994; Sim et al., 1994; Ranjan et al., 1999). EBA-175 and EBA-140 binds to Glycophorin B and in the case of EBA-175 the extracellular part of the molecular structure has been solved (Tolia et al., 2005), this is discussed further below. The precise localization and functionality of the Rh family remains open questions. It has been suggested that the Rh proteins perform functions similar to or in parallel with those of the DBL ligands (Triglia et al., 2005). The PfRh family was identified as homologs of the rhoptry proteins in *P. vivax* (Galinski et al., 1992) which are important in host cell binding and selectivity. While the DBL and PfRh proteins are important in merozoite invasion it is clear that they are not essential. All coding genes can be disrupted without affecting asexual growth rate (Duraisingh et al., 2003; Gilberger et al., 2003a; Maier et al., 2003; Stubbs et al., 2005). They each mediate invasion through different receptors and thereby they give rise to different invasion pathways amply demonstrated both in vitro adapted strains and in field isolates (Duraisingh et al., 2003).

1.4.1.4 Erythrocyte entry

The entry phase of merozoite invasion is again a rapid and vigorous process. After apical attachment an ultrastructural dense zone appears beneath the region of contact (Aikawa et al., 1978). The precise composition of this tight junction has not been clarified. The parasite may discharge mediators into the erythrocyte to assist in the invasion: albeit none has yet been identified. Movement into the host cell is based on a distinctive acto-myosin motor which together with accessory proteins is connected externally and internally to both the merozoite and the erythrocyte (King 1988; Webb et al., 1996; Pinder et al., 1998; Chaparro-Olaya et al., 2005). The link between the motor complex and the merozoite surface has not been identified; however the trombospondin related apical protein (TRAP) fills this role in invading sporozoites (Sultan et al., 1997; Kappes et al., 1999). A moving junction follows the rim of the merozoite as it enters the host cell. Many of the surface proteins are swept away from the merozoite as the moving junction advances over the surface (Ladda 1969; Bannister et al., 1975; Aikawa et al., 1978; Mitchell et al., 1988) and specific protease activity on the surface of the merozoite is essential in this process. This has mostly been studied in the processing of MSP-1 into the p19 and p33 fragments (Holder 1988; Holder 1994). The invagination of the erythrocyte membrane caused by the merozoite is converted during invasion to a PVM through insertion of rhoptry proteins into the erythrocyte cell wall (Bannister et al., 1975; Aikawa et al., 1978; Bannister et al., 1989). Much evidence supports the fact that erythrocyte membrane proteins are cleared from the developing PVM (McLaren et al., 1979; Aikawa et al., 1981; Ward et al., 1993). There is however conflicting evidence whether parasite lipids are also inserted in the PVM or not (Mikkelsen et al., 1988; Sjoberg et al., 1991; Haldar et al., 1992; Dluzewski et al., 1995). Recent work by the Haldar group indicates that there is selective insertion of specific phospholipids in the PVM in a DRM dependent manner described below (Murphy et al., 2007). When the merozoite is inside its new host cell, it is entirely enclosed by the PVM, with the junction remaining for a period of time at the posterior end of the parasite. When fusion is complete a fourth set of vesicular organelles come into play, the so called microspheres or dense bodies. These fuse with the plasmalemma and discharge their content into the PVM vacuolar space (Bannister et al., 1975) resulting in a coincidal distortion of the PVM. The ring infected erythrocyte surface antigen (RESA) was the first dense granule protein to be described (Kilejian 1980; Aikawa et al., 1990; Culvenor et al., 1991), the function remains unknown as are the functions of the other few dense body proteins described.

1.4.2 Trophozoite development in the erythrocyte host cell

The maturation of the parasite within the erythrocyte involves a multitude of metabolic pathways and precise timing of gene expression and silencing. In order to acquire sustenance the parasite digests the abundant erythrocytic haeme and stores the toxic metabolites in a centrally placed food vacuole. The parasite has to go through mitosis or meosis in corresponding cell cycle phases that are controlled by a variety of intra- and extracellular stimuli. Most of these implicate phosphorylation cascades, which lead to the modulation of the activity of cell cycle regulators. Successful growth and multiplication of the parasite require an active and temporally controlled metabolic program leading to the duplication of the structural components. The specifics of

trophozoite metabolism are beyond the scope of this thesis and will not be addressed further.

1.4.3 Protein trafficking

1.4.3.1 Passage into the parasite vacuole

Infection of human erythrocytes by *P. falciparum* results in complex membrane sorting and signaling events in the mature erythrocyte. Although major host proteins seem to be excluded from the membranes in the PVM at formation, proteins resident in erythrocyte detergent-resistant membrane (DRM) rafts are trafficked to the malarial vacuole. Rafts are conceived as clusters of free floating islands of proteins and lipids, held together by a cholesterol rich environment (Simons et al., 1997; Brown et al., 1998; Friedrichson et al., 1998). When the erythrocytes are depleted of cholesterol, no detectable effect is seen on major erythrocyte membrane function, but it disrupts DRM rafts and blocks malarial infection (Samuel et al., 2001). Signaling via erythrocyte β_2-adrenergic receptor (β_2-AR) and guanine nucleotide-binding protein (Gαs), both of which are raft proteins, regulates entry of *P falciparum* (Harrison et al., 2003). These data suggest that DRMs and their resident proteins may play a role in the induction and formation of the PVM in the erythrocyte. Upon malarial invasion, the PVM becomes selectively cholesterol-enriched, and 15 of the known raft proteins are internalized to the PVM (flotillin-1 and -2, Gαs, β_2-AR, AQP1, Duffy, CD55, CD58, CD59, scramblase). Most of the abundant erythrocyte membrane proteins are not internalized to the PVM (ie, glycophorins A and C, cytoskeleton-associated band 3, and others) (Murphy et al., 2004). An interconnected tubovesicular network (TVN) that extends from the parasitophorous vacuole membrane has been described and reported to be involved in the uptake of exogenous nutrients and drugs (Lauer et al., 1997).

1.4.3.2 Passage from the parasite

Proteins transported from the malarial parasites must follow complex route. After crossing the parasite's own plasma membrane, proteins pass the parasitophorous vacuolar membrane (PVM), then further on either into the host cytoplasmic space or through it to the erythrocyte surface. The study of the export of parasite proteins into the erythrocyte has relied heavily on microscopic visualization assays. Ever since Georg Maurer 1902 and others (Marchiafava et al., 1883 ; Schüffner 1899; Maurer 1902) discovered the reticulated vesicular network that bears Maurers name, malariologists have quizzed over the importance of these structures. It is now appreciated as a novel type of secretory organelle and has been further visualized by EM (Langreth et al., 1978; Atkinson et al., 1990; Wickert et al., 2003; Wickert et al., 2004) and 3D electron tomography (Hanssen et al., 2008). Because the erythrocyte lacks the secretory apparatus found in other eukaryotic cells, the parasite cannot use any host mechanism for protein sorting and transport. Therefore, it must establish de novo a secretory system in the host cell cytoplasm. There is growing evidence to suggest that the Maurer's clefts are involved in protein trafficking. Fluorescence and electron microscopic studies using antibodies against exported proteins, such as giant protein Pf332 and immuno-variant antigens including PfEMP1, RIFIN, and SURFIN (surface-associated interspersed gene encoded protein) have shown that these proteins transiently associate with the Maurer's clefts on their way to the host erythrocyte plasma membrane (Hinterberg et al., 1994; Wickham et al., 2001; Kriek et al., 2003;

Wickert et al., 2003; Haeggstrom et al., 2004; Winter et al., 2005). Disrupting gene function of residential proteins of Maurer's clefts, such as PfSBP1 (Cooke et al., 2006; Maier et al., 2007), abrogates export of PfEMP1 to the erythrocyte surface, suggesting that the Maurer's clefts, including the residential factors (which may aid export or be involved in maintaining the structure of the clefts), are required for directing exported proteins across the host cell cytoplasm to the erythrocyte plasma membrane. Interestingly, Maurers cleft are seen as small single, vesicle like structures in long-term propagated parasites while they are large and abundant in newly *in vitro* adapted field isolates parasites or in parasites that are highly up-regulated for VSA-expression (Haeggstrom et al., 2004). Further, studies indicate that Maurer's clefts are anchored to the host erythrocyte cytoskeleton. Maurer's clefts are often found underneath knobs in close proximity to the inner leaflet of the erythrocyte plasma membrane (Etzion et al., 1989; Hinterberg et al., 1994; Waterkeyn et al., 2000; Wickham et al., 2001). Moreover, Maurer's clefts stay attached to erythrocyte ghosts after lysis by osmotic shock or merozoite release (Martinez et al., 1998; Blisnick et al., 2000). The exact mode of transport to and from the Murers clefts is not clear. Two different, though not necessarily mutually exclusive, models have been proposed: trafficking by lateral diffusion along a continuous membrane network from the PVM to the erythrocyte plasma membrane and trafficking by vesicles. A common denominator for the parasite proteins designated for transport to the erythrocyte is that they contain a bipartite trafficking signal. The first part of the signal, the N-termainal signal sequence, is necessary for export into the parasitophorous vesicle (Wickham et al., 2001; Lopez-Estrano et al., 2003), and the second motif targets proteins for onward transport into the erythrocyte proper, called the *Plasmodium* export element (PEXEL) (Marti et al., 2004) or the vacuolar transport signal (VTS) (Hiller et al., 2004). Although PEXEL/VTS functions as a signature for a large number of exported proteins, this motif is by no means definitive. Several proteins, for which strong experimental evidence supports that they are exported, lack the PEXEL/VTS motif (Hiller et al., 2004; Marti et al., 2004).

1.4.4 Erythrocyte membrane modification and pathogenesis

As soon as the malaria parasite commences modification of the erythrocyte and the erythrocyte membrane it will begin to interact with the outside environment and the host immune system. A number of pathogenic features become evident at this point. *P. falciparum* infections are characterized by the fact that few mature trophozote stage parasites can be found in the peripheral circulation, even in patients with a very heavy parasite load. Identification of mature (>18 hour) trophozoite and shizont stages in the peripheral blood is actually a pathognomonic observandum of severe disease (Beeson et al., 2002). The mature forms can be localized in the capillaries and venules of vital organs and the cause of the phenomenon is the adherence of parasitized cells to vascular endothelium (Trager et al., 1966; Udeinya et al., 1981). This process is termed sequestration. Albeit, exceptions to the dogma have been seen in two studies, the first describes ring stage sequestration in cerebral malaria patients in autopsy material (Silamut et al., 1999) and the second shows the same in placenta of one otherwise healthy woman with no peripheral parasitemia (Muehlenbachs et al., 2007). Interestingly, a recent study indicates than not only mature stage IEs are retained in the spleen but also ring stages. In this material more than 50% of rings were also retained

in an *ex vivo* spleen perfusion assay (Safeukui et al., 2008). This suggests that there may exist subpopulations of early stage IEs that are less deformable and more prone to splenic clearance. The implications of sequestration in relation to clinical symptoms seen in CM and pregnancy associated malaria have been discussed above. The evolutionary advantage of sequestration may well be twofold; by lodging in the hypoxic capillary vessels the parasite ensures optimal growth and at the same time avoids splenic clearance. It is believed that the complicated pathology seen in severe *P. falciparum* disease is intimately associated to the parasites ability to sequester. The parasite displays several different binding phenotypes; cytoadhesion denotes the adhesion to endothelial cells and rosetting denominates the binding of parasitized red cells to uninfected ones. Furthermore the parasites may demonstrate aggregation of infected cells to each other into what is termed giant rosettes in a platelet mediated manner (clumping) (Wahlgren et al., 1995) or without platelets involved (autoagglutination, giant-rosetting (Carlson et al., 1990b; Roberts et al., 1992). All these phenotypes were initially described *in vitro* and are thought to reflect parasite conduct in the patient. An *in vivo* model of sequestration was established by this lab where radio-labeled human infected erythrocytes were injected into the tail vein of rats and rhesus monkeys (Pettersson et al., 2005; Moll et al., 2007). Parasites with multiadhesive phenotypes sequestered in the deep circulation and binding could be abrogated through blocking of variable surface antigens (VSAs). Recently real time *in situ* assessment of sequestered parasites in patients has been made possible through orthogonal polarization spectral (OPS) imaging microscopy of rectal mucosa (Dondorp et al., 2008a). In this study patients with severe falciparum malaria showed extensive microvascular obstruction that was proportional to the severity of the disease.

1.4.5 Structures on the surface

The binding interactions are a product of major alterations of the surface of the infected erythrocyte. During the 12-14 first hours of intraerythrocytic development there are no noticeable ultrastructural changes in the erythrocyte cytoplasm. In the following hours a change in permeability of the erythrocyte membrane takes place (Homewood et al., 1974; Ancelin et al., 1991; Staines et al., 1998; Staines et al., 2002). The shape and deformability of the erythrocyte alters and electron dense protrusions known as knobs appear on the outer face of the erythrocyte membrane (Trager et al., 1966; Cranston et al., 1984; Nash et al., 1989; Paulitschke et al., 1993). The knobs are compact 100nm structures positioned directly below the surface of the IE membrane. Knobs consist predominantly of the knob-associated histidine-rich protein (KAHRP) (Kilejian 1979; Culvenor et al., 1987; Pologe et al., 1987), assembling on the cytoplasmic face of the membrane. KHARP is believed constitute a stable platform for presentation of VSAs on the erythrocyte exterior. Indeed KHARP has been shown to interact with the ATS domain of PfEMP1(Waller et al., 1999), the main sequestration mediator described in detail below. It also interacts with spectrin and f-actin (Kilejian et al., 1991), suggesting a stabilizing function. Another known knob component is the mature parasite-infected erythrocyte surface antigen (MESA) (Coppel et al., 1986; Howard et al., 1987). MESA mediates anchoring of the knob complex to the erythrocyte membrane skeleton by binding non-covalently to protein 4.1, a key host protein in regulating the membrane mechanical properties of the red cell (Lustigman et al., 1990). MESA is not required for cytoadherence under static conditions (Petersen et al., 1989); however, its binding to

protein 4.1 is crucial for parasite survival (Magowan et al., 1995). A third knob member is the *P. falciparum* membrane protein 3 (PfEMP3) (Pasloske et al., 1993). The function of this protein is not known, it does not cluster to the same extent to knobs as KHARP, but it has been suggested to aid in the rigidification of the IE membrane (Glenister et al., 2002).

1.4.6 Cytoadherence

1.4.6.1 CD36

The term cytoadhesion encompasses all the IE binding events to vascular endothelium, including binding to the placental syncytiotrophoblasts. A number of endothelial receptors have been identified as targets for the IE, including CD36 (Barnwell et al., 1989), intercellular adhesion molecule-1 (ICAM-1) (Berendt et al., 1989), chondroitin-4-sulfate (CSA) (Robert et al., 1995; Rogerson et al., 1995), VCAM and E-selectin (Ockenhouse et al., 1992), PECAM-1/CD-31 (Treutiger et al., 1997), heparan sulfate (HS); (Vogt et al., 2003) and P-selectin (Udomsangpetch et al., 1997). In vitro, CD36 is the most frequent target of strains from patients with mild as well as severe *P. falciparum* malaria (Turner et al., 1994). There is subsequently no strong evidence for a specific role for CD36 in severe disease, since almost all IE bind to the receptor. Still it should be important in enhancing binding. Among children with severe malaria in Africa, parasite binding to CD36 was inversely related to disease severity (Rogerson et al., 1999); another study found that CD36 binding was equivalent between parasitized erythrocytes derived from CM patients or community controls (Newbold et al., 1997). The region recognized by IE on CD36 maps to a hydrophobic segment located at residues 145 to 171 of the CD36 receptor (Baruch et al., 1997). This was elucidated through production of CD36 peptides of varying lengths which were tested for the ability to block binding of IE to CD36.

1.4.6.2 ICAM-1

A second important receptor is intercellular adhesion molecule 1 (ICAM-1), a 90-110kDa polypeptide comprising five Ig like homology domains in the extracellular portion of the molecule, a transmembrane segment and a short cytoplasmic tail (Staunton et al., 1988), ICAM-1 is present on the surface of endothelial cells and monocytes. The expression of ICAM-1 is upregulated by the proinflammatory cytokines IL-1β, TNFα and IFNγ as well as endotoxin/LPS (Dustin et al., 1986; Berendt et al., 1989). Binding by IE to vascular endothelium can by itself upregulate the expression of ICAM-1 (Udeinya et al., 1993). In several studies ICAM-1 and CD36 have shown to act synergistically in increasing the binding to endothelial cells (McCormick et al., 1997; Yipp et al., 2000). Furthermore, a recent study shows that intracellular signaling cascades are activated through IE binding to ICAM-1 and CD36, and that the activation is parasite strain dependent (Jenkins et al., 2007). ICAM-1 is a candidate for IE binding to brain endothelium (Turner et al., 1994). In this study fatal malaria was associated with widespread induction of endothelial activation markers in the brain, with significantly higher levels of ICAM-1 and E-selectin expression on vessels in the brain. Also in one study binding to this receptor was elevated in parasites collected from patients with cerebral malaria, although this did not reach significance (Newbold et al., 1997). The exact regions of ICAM-1 that interact with IE have been mapped in three studies to the BED 'side' of the N-terminal Ig-like domain (Berendt et

al., 1992; Ockenhouse et al., 1992; Tse et al., 2004), implicating residues G15, L18, T20 and L43 in close vicinity to each other in the solved crystal structure. The latter study also showed that binding affinities to different ICAM-1 mutants varied in different parasite lines suggesting that there are subtle differences in the contact residues used by different parasites.

1.4.6.3 CSA and other placental receptors

As previously mentioned Chondroitin sulfate A (CSA) is the main receptor involved in placental malaria. CSA is a glycosamino-glycan linked to the cell surface via a membrane-associated protein. In 1996 Duffy and Fried proposed a molecular model to placental sequestration and PAM pathogenesis. It stated that the placenta presents surface receptors in the vascular bed that is not accessible for parasite adhesion elsewhere. Serum immunoglobulin G (IgG) from multigravid women living in areas of endemicity has been shown to block adhesion of placental or CSA-selected parasites collected from different continents (Fried et al., 1996; Beeson et al., 1999; Maubert et al., 2000). Adhesion-blocking antibodies are not detected in males or women before first pregnancy. This pattern of naturally acquired immunity is consistent with repeated exposure to a finite number of antigens during pregnancy that are not seen in childhood infections, raising expectations that a vaccine can be developed. Further, non-immune-immunoglublins have been suggested to act as bridges between the VSA PfEMP1 and the placenta and thereby assist CSA in placental adhesion (Flick et al., 2001; Rasti et al., 2006). Hyaluronic acid (Beeson et al., 2000; Rasti et al., 2006) has been described as an additional receptor relevant to PAM although there is controversy regarding these last two findings (Rowe et al., 2002; Creasey et al., 2003; Muthusamy et al., 2007; Ghumra et al., 2008). The molecular binding events will be further described in the PfEMP1 section of the thesis.

1.4.6.4 Other surface receptors

Other endothelial receptors studied include PECAM-1/CD31 and Thrombospondin (TSP) and heparan sulfate. PECAM-1 is a highly glycosylated 130kD member of the immunoglobulin superfamily that is expressed on the surface of certain intravascular cells such as nutrophils, platelets, monocytes and T-cell subsets. It is furthermore extensively expressed on the surface of endothelial cells (Newman 1997). It consists of six extracellular immunoglobulin-like homology domains with nine potential N-linked glycosylation sites and a relatively long cytoplasmic tail. Binding of IE to PECAM-1 has shown to involve the first four domains of PECAM-1 and that PECAM-1 binding occurs independently of the rosetting phenomenon (Treutiger et al., 1997). Thrombospondin (TSP) is a large protein consisting of three identical 140–kDa chains linked to each other by inter-chain disulfide bonds. It is found in the extracellular matrix, in platelet granules, as well as on the surface of macrophages, melanoma cells and a variety of endothelial cells. TSP binds to CD36, CD47 and IE, and plays roles in platelet clotting and regulation of angiogenesis. Most clinical isolates and laboratory lines bind to TSP (Roberts et al., 1985; Baruch et al., 1996; Heddini et al., 2001). It has been suggested however that TSP binding can be separated from other cytoadherence mechanisms (Newbold 1999). Furthermore VCAM-1, E-selectin and P-selectin display binding *in vitro,* however they seem to be poorly recognized by *P. falciparum* field isolates (Ockenhouse et al., 1992; Udomsangpetch et al., 1997).

1.4.7 Rosetting

P.falciparum infected red cells can adhere to uninfected red cells to form spontaneous rosettes (Wahlgren 1986; Udomsangpetch et al., 1989a). This was first described *in vitro* but later confirmed in *ex vivo* blood samples examined directly after sampling (Carlson et al., 1990a; Ho et al., 1991; Wahlgren et al., 1992; Chotivanich et al., 1998). Rosettes have also been observed in post-mortem histological specimens (Riganti et al., 1990; Pongponratn et al., 1991). Several studies indicate that rosettes are strong enough to withstand the shear forces experienced in vivo (Nash et al., 1992; Chu et al., 1997; Chotivanich et al., 2000). Rosetting becomes apparent in mature stages of the parasite intraerythrocytic life-cycle, and it typically commences 16-18 hours post entry. Rosetting persists through schizogony and disappear upon bursting (Treutiger et al., 1998) (Paper IV) and appears again in the following cycle. The capacity to form rosettes has been observed in all of the malaria species that infect humans, except for *P. knowlesi* which has not yet been examined in this respect (Handunnetti et al., 1989; Udomsangpetch et al., 1989b; Udomsanpetch et al., 1995; Angus et al., 1996; Lowe et al., 1998). It was realized early on that rosetting is a phenotype that varies between *P. falciparum* isolates (Wahlgren et al., 1990). This led to the finding that variable surface antigen family PfEMP1 is the agent mediating rosetting in *P.falciparum* (Rowe et al., 1997; Chen et al., 1998). Median rosetting rates in field isolate parasites have also in many studies been correlated to severe disease in the patient (Carlson et al., 1990a; Treutiger et al., 1992; Ringwald et al., 1993; Rowe et al., 1995; Newbold et al., 1997) and more. In the majority of these studies, rosetting is associated with cerebral malaria and other severe malaria syndromes such as severe anemia, respiratory distress and prostration. A number of different erythrocyte surface ligands have been described to be involved in rosetting. These include heparan sulphate (HS) -like glycosaminoglycans (GAG), ABO blood group antigens, complement receptor-1 (CR1) and CD36. Different parasite strains have been shown to have variable sensitivity to rosette-disrupting agents such as heparin, heparan sulfate, fucoidin and dextran sulfate (Udomsangpetch et al., 1989b; Carlson et al., 1990a; Rogerson et al., 1994; Rowe et al., 1994; Barragan et al., 1999). Rosettes resistant to heparin are often efficiently disrupted by other GAGs, which suggest that *P.falciparum* can use a wide repertoire of glycan structures as ligands. Heparan sulfate has indeed been found on the surface of normal red cells (Vogt et al., 2004) and there is evidence to advocate that modified heparin without anticoagulant activity can be used as a de-sequestration and anti-rosetting agent (Vogt et al., 2006).

1.4.7.1 Blood group AB0 antigens

A preference to either group A or B red blood cells in rosettes has been reported in *in vitro* parasite strains (Carlson et al., 1992a; Treutiger et al., 1999) and field isolates (Carlson et al., 1992a; Udomsangpetch et al., 1993; Rowe et al., 1995; Chotivanich et al., 1998; Barragan et al., 2000b; Rowe et al., 2007). This preference is reflected in that the rosettes are larger and can withstand higher shear stress. The sensitivity of rosettes to disruption with heparin, HS or other GAGs is drastically diminished when the parasite is cultured in the preferred blood group (Carlson et al., 1992b; Barragan et al., 2000b). *P. falciparum* rosettes are able to form in group O cells, but these are usually weaker and smaller (Carlson et al., 1992b; Somner et al., 2000). Recent work in Mali shows that children with blood group O are protected against severe malaria through

the mechanism of reduced *P. falciparum* rosetting (Rowe et al., 2007). In line with this other studies have correlated ABO groups to severe/cerebral malaria (Hill et al., 1992; Lell et al., 1999; Pathirana et al., 2005; Rowe et al., 2007; Fry et al., 2008). This was confirmed by a recent meta-analysis, which found group A associated with severe disease and blood group O with milder disease (Loscertales et al., 2007).

1.4.7.2 CR-1

Studies on heparin insensitive rosetting parasite strains led to the finding that CR1 acts as a rosetting ligand. Rosetting in specific strains is impaired in erythrocytes with low level of CR1, is inhibited by soluble CR1 (Rowe et al., 1997) and by a specific anti-CR1 monoclonal antibody (Rowe 2000). CR1 is an immune regulatory molecule that is expressed on the surface of erythrocytes, some peripheral blood leukocytes, glomerular podocytes, and follicular dendritic cells (Ahearn et al., 1989). CR1 binds to the activated complement components C3b and C4b, and thereby cause clearance of immune complexes from the circulation, enhances phagocytosis, and regulates complement activation (Ahearn et al., 1989). A polymorphism in the *cr1*gene that causes erythrocyte CR1 deficiency occurs at extremely high frequencies in malaria endemic regions of Papua New Guniea and the genotype is protective against severe malaria (Cockburn et al., 2004). Studies in Africa have also suggested that red blood cell CR1 levels can influence the disease outcome (Waitumbi et al., 2000; Stoute et al., 2003; Waitumbi et al., 2004). The region of CR1 required for rosetting with parasite clone R29 maps to the C3b binding site of CR1 which corresponds to the middle part of the molecule (Rowe et al., 2000).

1.4.7.3 Serum proteins

Serum proteins have been shown to be essential for the formation of rosettes both in *in vitro* strains as well as field isolates (Treutiger et al., 1999; Rogerson et al., 2000; Somner et al., 2000). This was first shown by the demonstration of human immunogloubulins in fibrillar strands connecting infected and uninfected red blood cells (Scholander et al., 1996). There is evidence that IgM (Scholander et al., 1996; Clough et al., 1998; Somner et al., 2000) and IgG (Flick et al., 2001) (Paper IV) as well as fibrinogen and vonWillebrand´s factor (Treutiger et al., 1999) partakes. By using domain-swapped Abs, mutant IgM molecules, and specific mAbs to IgM the Rowe group recently demonstrated that IE binding requires the Cμ4 domain of the IgM H chain, and that IgM polymerization is essential for binding. This region is homologous to the regions in IgG and IgA that are bound to by bacterial Fc-binding proteins. Furthermore, in the same study, multiple *P. falciparum* strains implicated in both severe childhood and pregnancy-associated malaria were shown to use the same binding site on IgM (Ghumra et al., 2008). The parasite ligands involved in binding are discussed further down.

1.5 ANTIGENIC VARIATION IN *P. FALCIPARUM*

To be able to understand the molecular mechanics of sequestration one has to also comprehend the role of variable surface antigens in pathogenesis. The ability of pathogens to sequentially express related but variant molecules on governed cell surfaces is a stratagem widely adopted by many infectious organisms. Antigenic variation provides an opportunity for the microorganism to establish a chronic infection

and maximize the possibility of transfer to other hosts. It seems that evolution favors these systems to maintain and transmit the pathogen within/from the host without killing it too swiftly. Examples are manifold and involve numerous phylae, such as *Borrelia spp* (Wilske et al., 1996), african trypanosomes (Turner 1999) and *Chlamydia* (Brunham et al., 1994). Antigenic variation carries certain common premises in most of these organisms. The antigens involved are encoded by large nonallelic gene families and the expression of the gene products switches in a regulated and timed order. The switching is either accomplished through DNA recombination or the activation/deactivation of promoters regulating individual genes in active expression sites. The antigens elicit strong humoral immune responses that show low cross-reactivity between individual VSA species and antigenic diversity is ensured through recombination and gene conversion. *P. falciparum* has the potential to express a vast repertoire of variant proteins on the surface of the infected red blood cell. In the eighties a strain specific component of *P.falciparum* infected erythrocyte (IE) surfaces was demonstrated to be a family of large polymorphic proteins termed Plasmodium falciparum membrane protein 1 (PfEMP1) (Leech et al., 1984). It was shown that presence of VSA on the surface of IE conferred the ability of the IE to bind to different cell surfaces (David et al., 1983). Ten years later the gene family encoding PfEMP was discovered and was termed *var* (Baruch et al., 1995; Smith et al., 1995; Su et al., 1995). Further studies proved the connection between surface adhesion and the sequestration phenomenon seen in the deep vasculature of *P. falciparum* infected patients (Newbold et al., 1997). Since then numerous cell-surface receptors have been mapped to PfEMP1 antigen-ligand interactions and these will be covered in greater detail.

Surface iodination experiments have also revealed a trypsin sensitive variant family with a two TM domain topology named RIFIN, encoded by the *repetitive interspersed family* (*rif)* genes (Fernandez et al., 1999; Kyes et al., 1999). This highly polymorphic gene family holds even more copies in the genome (~160) (Cheng et al., 1998) than the *var* family and the proteins are considerably smaller (30-45 kD). Transcripts of *rif* are maximally detected at the early pigmented trophozoite stage, i.e. approximately 18 hours after invasion (Kyes et al., 1999; Kyes et al., 2000). Up to this point, no biological function has been associated to the RIFINs, however IE surface recognition of RIFINs by cross-reactive antibodies from hyper-immune sera has been shown in naturally occurring infection (Abdel-Latif et al., 2002). The *rif* genes are comprised of two exons where exon I encodes a short 19 amino acid semi-conserved signal sequence followed by a -200 nucleotide (nt) intron. Exon II consists of a variable domain where two regions are predicted to be transmemebranous, one in the middle of the protein and the second located in the C-terminal end. The rif genes can further be subdivided into two sub-classes, group A and B (Gardner et al., 2002; Joannin et al., 2008) primarily depending on a 25 nt deletion in the group A genes. The importance of the different groups in relation to function is still unclear.

The RIFINs are structurally related to the Sub-Telomeric Variable Open Reading Frame (STEVOR) family. Although primary sequence differs quite substantially between the two families, they are still considered to have a common ancestry (Finn 2008). First reported as *7h8, stevor* was initially identified as an expressed sequence detected by a monoclonal antibody (Limpaiboon et al., 1991). There are 30-40 *stevor* genes per haploid genome, located to a large extent adjacent to rif genes in the subtelomeric regions of the chromosomes (Gardner et al., 2002). Recent studies have located the STEVOR proteins to the surface of the IE, however conclusive evidence is

lacking to whether the STEVORs are exposed on the IE surface or not (Abdel-Latif et al., 2004; Lavazec et al., 2007). (Blythe et al., 2008) also demonstrated that STEVORs are more highly expressed in patient isolates compared to *in vitro* strains, and that they could also be located to the apical end of merozoites. The STEVORs share localization with the SURFIN (Surface associated interspaced gene) family of proteins (Winter et al., 2005) in that it locates to the merozoite apex and IE surface. The SURFIN´s were identified by mass spectrometric analysis of peptides cleaved off the surface of live IEs with trypsin. The *surf* repertoire of genes consist in the 3D7 of a family of ten genes located within or close to the subtelomeres of five of the chromosomes, including three predicted pseudogenes. A fifth variant protein present on the surface of the IE; PfMC-2TM with 13 members in the 3D7 genome has been described by (Sam-Yellowe et al., 2004). The function of the STEVORs, PfMC-2TM and SURFINs remain as elusive as the RIFINs. A model where the *rif* and *stevor* genes are involved in merozoite immune evasion has been proposed.

1.5.1 Antigenic variation and PfEMP1

When it became apparent that PfEMP1 (and its encoding family of *var* genes) was the main ligand responsible for vascular adhesion and pathogenesis of *P. falciparum*, intense focus was shifted towards elucidating the molecular basis of the events observed in the patients. The complexity involved in this effort can easily be appreciated and has generated a veritable mountain of data. Both parasite and host factors have had to be considered, often isolated from each other because of methodological hurdles. The highly polymorphic *var* genes present a challenge not only in their inherent variability but also the complexity in which they are regulated. The human host too carries specific variable genetic and immunological traits that influence PfEMP1 interactions with surface receptors.

1.5.1.1 Genomic organization of var genes

The completion of the 3D7 *P.falciparum* genome sequence project not only opened a whole new era in the study of malaria research, it also provided a detailed map of *var* gene organization and primary structure. It confirmed earlier results where the genes had been tallied to about 60 per genome, located to the highly polymorphic chromosome end regions, or in a smaller number to central chromosome regions (Su et al., 1995; Rubio et al., 1996; Hernandez-Rivas et al., 1997; Gardner et al., 2002) (Figure 2A). The majority of the *var* genes, roughly two thirds, can be found adjacent to non-coding telomere repeat elements (TARE 1-6) usually in a tail to tail orientation with one or more *rif* genes in between or clustered together followed by a number of *rif* or *stevor* genes. Each of the fourteen chromosome ends typically contains one or two *var* genes each except for the right telomere of chromosome 14 that lack *var* genes. One third of the *var* gene repertoire locates to the central chromosomal domains, either in clusters of three to seven genes in a head to tail orientation or singly placed.

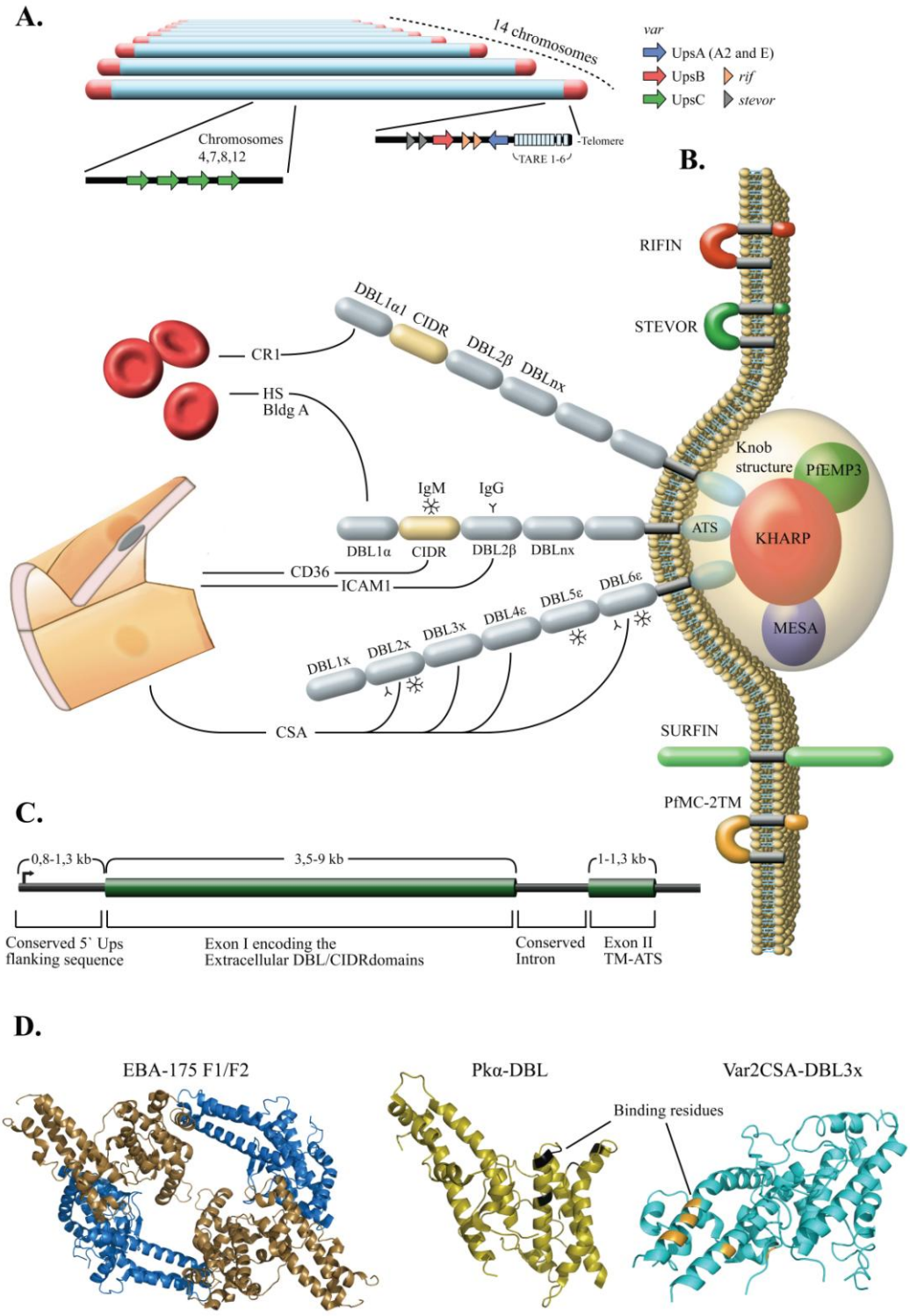

Figure 2: **A**. The genomic organization of *var* genes. Central UpsC *var* genes are located in the central regions of the chromosomes and UpsA and UpsB locate to the subtelomeric regions. **B.** PfEMP1 location on the IE membrane and the different binding affinities of the different DBL/CIDR domains. Group A, Group B/C and Var2CSA PfEMP1 proteins are exemplified from top to bottom. Other VSA proteins present on the IE surface are represented along the membrane. **C.** The configuration of *var* genes, a two exon architecture with the two promoter regions, the 5´-Ups and the conserved intron. The relative lengths of the regions are shown. **D.** The three determined DBL structures. The EBA-175 F1/F2 complex of four DBL domains illustrates the "handshake" orientation. Pkα-DBL and the Var2CSA domains are mirrored position-wise and the residues implicated in binding are drawn in other colors.

Comparison to other sequenced strains (HB3 and IT4) confirms that this organization is similar in genetically distinct strains from different geographical locations (Volkman et al., 2007). The chromosomal location and transcriptional orientation have been shown to correspond to the 5´-prime upstream coding sequence of the gene. The upstream groups have arbitrarily been termed as UpsA to E where UpsD has recently been re-annotated to UpsA2 (Kraemer et al., 2007) and is a subcategory of UpsA. The majority of the sub-telomeric genes that are transcribed towards the centromere belong to the UpsB group, albeit a few UpsB genes locate to the central parts of the chromosomes. Subtelomeric genes that are transcribed towards the telomeres mostly belong to the UpsA type. All centrally placed *var* genes univocally belong to the UpsC group. There is only one known exception, an UpsA *var* gene that is predicted to be in a central chromosome cluster in HB3 (Kraemer et al., 2007), but this may represent a sequence assembly artifact.

1.5.1.2 Structure of PfEMP1

The PfEMP1 proteins are very large multi-domain molecules with a size of ≈ 250-350,000 Da (Figure 2C.). The *var* genes have a two exon arrangement separated by a conserved intron, where exon I constitutes the majority of the gene and varies in size from about 3.5 to 9.0 kb. It encodes the extracellular multiple adhesion domains termed the Duffy binding like (DBL) and cysteine rich interdomain region (CIDR). The end tip of the protein consists of a N-terminal segment (NTS) and the adhesion domains can be interspersed with C2 interdomains, not present in every variant. Most PfEMP1 variants have a semiconserved protein head structure consisting of NTS-DBL1α-CIDR1 domains. The smaller (1.0 to 1.5 kb) exon II encodes a semiconseved amino acid terminal segment, which has proven to contain a C-terminal transmembraneous region (Smith et al., 2000b). Because of the relative high content of acidic residues in this domain it has been termed the acidic terminal segment (ATS) (Su et al., 1995). PfEMP1 sequences are as previously mentioned extremely variable, however careful sub-sectioning and comparison of the different PfEMP1 species has shown that there is a method to the seemingly endless variation. This partitioning is layered, and each group of genes can further be sub-grouped into smaller categories. This categorization has shown to be directly linked to the function and binding capabilities of each protein. Primarily the adhesion domains can be grouped according to sequence similarity into seven types of DBL domains (α, α_1,β, γ, δ, ε, and x) and four types of CIDR domains (α, α_1, β, and γ) (Smith et al., 2000b).

1.5.1.3 Genome parasite PfEMP1 organization

The PfEMP1 proteins in the 3D7 genome have been subjected to further classification into one of seventeen different protein architectural types based upon domain composition (Gardner et al., 2002; Kraemer et al., 2006) and divided into three major (A, B, and C) and two intermediate (B/A and B/C) groups on the basis of 5' upstream (Ups) sequence and chromosomal location (Gardner et al., 2002; Kraemer et al., 2003; Lavstsen et al., 2003) (Figure 2B.). In 3D7, *Var* group A consists of ten genes that have UpsA 5- upstream sequences and are located in sub-telomeric regions transcribed toward the telomere. Group A *var* genes mainly encode large PfEMP1s with complex multi-domain structure that usually contain one or two C2 inter-domains. The largest *var* group in 3D7, group B, consists of 22 genes sharing 5' UpsB region. All genes but

one are located in the telomeric region and are transcribed towards the centromere. The encoded proteins typically have the characteristic four-domain structure, DBLα-CIDRα-DBLδ-CIDR2. Group C are flanked by UpsC sequences and are located in central chromosomal regions. Group B/A genes are very similar in location and transcriptional orientation to group B genes, but are located further from the telomere. Furthermore, group B/C genes have an UpsB-like 5' flanking sequence, but are located in central chromosomal regions. These observations has led to the hypothesis that the B/A and B/C groups of genes are evolutionary intermediates between groups A and B, and groups B and C, respectively (Lavstsen et al., 2003).

1.5.1.4 Unique genes

Two unique types of genes have been characterized in all sequenced isolates which do not fit in the classification above. They both are associated to an individual upstream 5´-sequence and are unusually conserved across strains. The *var2CSA* genes are flanked by a 5´UpsE and have been linked to CSA binding, which makes them critical to the pathogenesis of pregnancy associated malaria. Although there is limited overlap of variant antigen repertoires between isolates, *var2CSA* is an exception. Most or all parasite isolates contain a *var2CSA* ortholog conserved at circa 75% amino acid identity across worldwide parasite isolates. Two copies of the gene can be found in HB3 where the two copies are genetically distinct, while IT4 and 3D7 only carries one copy each (Kraemer 2007). The Var2CSA protein is built up of six DBL domains, DBL1x-DBL2x-DBL3x-DBL4ε- DBL5ε- DBL6ε, and the domain composition is conserved in all var2CSA genes sequenced to date. The second gene type carries the UpsA2 (UpsD) flanking sequence and was initially named *var1CSA* as its DBL3 γ domain was shown to mediate CSA adhesion (Buffet et al., 1999; Vazquez-Macias et al., 2002), later publications has termed it *varCOMMON* (Winter et al., 2003) or simply *var1* (Lavstsen et al., 2003). A survey of field isolates from geographically different locations shows that the 3D7 version of this gene is hyper-conserved in many isolates from malaria endemic areas (Kyes et al., 2003; Winter et al., 2003). In 3D7 the gene (PFE1640w) is truncated and this seems to be the case in the field isolates that harbor the gene (Winter et al., 2003). In addition it shows an unusual stage specific transcription pattern in lab strains (Kyes et al., 2003) as well as in *in vitro* adapted field isolates (Paper III). The function of the var*1CSA/varCOMMON* genes is not known at this point. A third gene that falls partly outside of the common classification frame is the var3 gene. It is flanked by an UpsA sequence, but it only consists of one DBL domain. Remarkably, homologs of *var1CSA* and *var2CSA* are present in the chimpanzee malaria parasite *Plasmodium reichenowi* (Trimnell et al., 2006), although evolutionary divergence is believed to have occurred 6-10 million years ago (Ayala et al., 1999).

1.5.2 DBL/CIDR tertiary structure

The DBL domains are members of a larger erythrocyte binding-like (EBL) superfamily of proteins. The EBLs include not only the DBL domains of Pf EMP1, but also other proteins believed to be involved in erythrocyte invasion, among others: EBA-140 (Mayer et al., 2001; Thompson et al., 2001; Narum et al., 2002); EBA-181 (Gilberger et al., 2003b), MAEBL (Blair et al., 2002), EBA-175 (Camus et al., 1985; Adams et al., 1992; Klotz et al., 1992); Pf332 (Mercereau-Puijalon et al., 1987; Mercereau-Puijalon

et al., 1991; Moll et al., 2007) and also in other species such as Pkα-DBL of *P. knowlesi* (Miller et al., 1975; Miller et al., 1976). Three DBL domains as well as parts of CIDR have been structurally determined and these discoveries has had a large impact on our understanding of DBL:receptor interactions. The first to succeed in this was Tolia and co-workers, who crystallized the extracellular tandem DBL domain of the EBA-175, which has been implicated in merozoite attachment (Tolia et al., 2005) (Figure 2D.). The domain was expressed in a *pica pastoris* expression system, and co-crystallized with the α-2,3-sialyllactose part of the Glycophorin B receptor. The overall structure resembles an elongated molecule, comprised primarily of α helicies, with two antiparallel β hairpins and several bound sulfate molecules. The (F1 and F2) tandem DBL domain crystallized as a dimer, where two symmetrically related molecules interacts extensively with each other in an antiparallel orientation resembling a handshake. This creates in effect a mega-structure involving four DBL molecules with two channels in the middle of the complex. Six glycan binding sites were identified in the structure with two α-2,3-sialyllactose molecules bound to each complex one in the vicinity to each cannel. The binding of α-2,3-sialyllactose involves both monomers indicating that dimerization is essential for receptor recognition.

The following year the monomeric structure of Pkα-DBL was presented (Singh et al., 2006). It has poor sequence conservation to the F1/F2 DBLs from EBA-175 but retains a similar overall structure. Pkα-DBL binds the Duffy antigen receptor for chemokines (DARC) in the simian host, however earlier domain deletion (Singh et al., 2003) and site-directed mutagenesis studies (VanBuskirk et al., 2004; Hans et al., 2005) indicate that binding occurs on the opposite face of the molecule compared to the EBA-175 F1/F2 .

Furthermore, the M179 subdomain of CIDR was structurally determined by Klein and coworkers. M179 represents the CD36 binding portion from the PfEMP1 of the Malayan Camp (MC) parasite strain. MC179 constitutes about 65% of the MC CIDR1α domain and is composed of a bundle of three helices connected by loops and three additional helices. The CIDR1α three-helix bundle exhibits less than 20% sequence identity with the three-helix bundles of Duffy-binding like (DBL) domains, but the two kinds of bundles are almost structurally identical in a comparison between M179 and EBA-175/F2 as well as Pkα-DBL. It is predicted that the PfEMP1 molecule is a polymer of three-helix bundles elaborated by a variety of connecting helices and loops. Interestingly, M179 crystallizes as a dimer in the same handshake manner as EBA-175, and the hypothesis is that the connecting helices of the MC179 portion of CIDR1α domains bind another molecule, either a PfEMP1 domain or a ligand (Klein et al., 2008). Recently the 3D structure of the CSA binding DBL3x domain of var2CSA was determined (Higgins 2008; Singh et al., 2008) and it will be discussed further down.

1.5.3 PfEMP1 diversity and function

Subtelomeric VSA genes can be found in not only in *P. falciparum* but also in *P. vivax* (*vir*) (del Portillo et al., 2001) and *P. richenowi* (*var* homologues) (Jeffares et al., 2007). This suggests that the genomic location may be important to DNA diversification. Furthermore, FISH studies have shown that *P. falciparum* chromosomes are structurally positioned in the nucleus. Telomeric *var* genes locate to the nuclear periphery forming “boquets” of four to seven chromosome ends (Freitas-Junior et al., 2000; Frank et al., 2007). Also centrally placed group C var genes locate

to the nuclear periphery (Ralph 2005). It has been proposed that DNA exchange takes place in these boquets (Kraemer 2003, Scherf 2004), where the proximity of the genes to each other facilitate recombination. Sequence comparisons and restriction fragment length polymorphism analysis of parasite crosses as well as population studies indicate that both small (100-200 nucleotide) and larger recombination events contribute to *var* gene evolution (Ward et al., 1999; Taylor et al., 2000b; Bull et al., 2005; Barry et al., 2007; Frank et al., 2007; Kraemer et al., 2007). Further confirmation of the importance of recombination in maintaining *var* gene diversity derives from the Su group, which showed *in silico* that the middle and sub-telomeric regions of the chromosomes are highly prone to recombination events (Mu et al., 2005; Mu et al., 2007).

1.5.3.1 CIDR:CD36

A number of specific domains, carbohydrates and proteins, have been mapped to binding to different PfEMP1 domains (Figure 2B.). One of the most thoroughly described is the interaction between the host receptor CD36 and CIDR. The region within PfEMP-1 responsible for CD36 binding was initially mapped in the Malayan Camp (MC) line of *P. falciparum* to a 179-amino-acid fragment (MC-r179) within the central M2 region of the CIDR domain (Baruch et al., 1997) and confirmed using other parasite lines (Robinson 2003). Comparison of various CIDR domains from several strains of *P. falciparum* showed that this cysteine-rich "minimal" binding domain had a conserved sequence, including the presence of five cysteine residues forming the motif $CX_8CX_3CX_3CXC$. Recent work has shown that binding is mediated close to the C-terminus, while the larger N-terminal part of CIDR is essential for correct folding of the protein, a 60-amino-acid region located at the C terminus mediates binding to CD36. This was carried out through site directed mutagenesis (Mo et al., 2008). CIDR domains from different PfEMP1 groups also demonstrate differential binding affinity to CD36. On the basis of the 3D7 genome sequence CIDR domains from 50 *var* genes were expressed on the surface of cos-7 cells and tested for CD36 binding. The results indicate that CIDR domains from group B and C var genes bind to CD36 but group A var genes show no binding, due either to primary sequence differences in CIDR1 in the UpsA-type-associated PfEMP1 (Robinson et al., 2003) or complete lack of CIDR domains in type 3 *var* and *var2CSA*. Insights gained from the MC179-CIDR1α crystal structure indicate that M179 acts as whole, and only large changes in the domain affects binding to CD36 (Klein et al., 2008).

1.5.3.2 DBLβ-C2:ICAM-1

As with CD36, work has progressed to characterize ICAM-1 binding to PfEMP1. Three different PfEMP1 variants from the IT4/25/4 (IT4) parasite isolate and one from an Indian field isolate (JDP8) have been shown to bind ICAM-1 (Berendt et al., 1989; Ockenhouse et al., 1991; Gardner et al., 1996; Chattopadhyay et al., 2004). Of interest, all four ICAM-1 binders use a DBLβ-C2 tandem domain for binding, and the three ICAM-1 binders from the IT4 isolate all have UpsB type promoters (Smith et al., 2000a; Chattopadhyay et al., 2004; Springer et al., 2004). In a recent extesive study ICAM-1 binding was mapped to DBLβ-C2 domains in of the IT4 PfEMP1 repertoire using the same cos-7 cell expression approach as with the 3D7/CIDR :CD36 study mentioned above (Howell et al., 2008). Relatively few DBLβ-C2 domains bound to ICAM-1, (7 out of 21 domains tested). These were all of either group B (6) or C (1) *var* genes, none of the group A *var* genes showed recognition. Two of the UpsB binders

were mapped to subtelomeric locations (IT4var14 and IT4var31) and two to central chromosomal locations (IT4var27 and IT4var1) (Kraemer et al., 2007). Site directed mutagenesis was in the same study utilized to map sequence regions of interest to binding. Results indicate that putative glycan binding sites of DBLβ-C2 are involved in the interaction. 3D-Modelling using the EBA-175 F2 and Pkα-DBL structures as templates indicate that binding occurs on the face that corresponds to F1/F2: α-2,3-sialyllactose binding in EBA-175 (Howell et al., 2008).

1.5.3.3 Var2CSA:CSA

The PfEMP1:CSA interaction is very well characterized to date. Multiple lines of evidence link the *var* gene *var2CSA* to placental malaria. *Var2CSA* transcription is highly upregulated in parasites from infected placentas (Tuikue Ndam et al., 2005; Duffy et al., 2006) and pregnant women exposed to malaria infection acquire antibodies to recombinant *var2CSA* proteins suggesting this protein may be an important target of protective immunity (Salanti et al., 2004). Furthermore, the ability of infected erythrocytes to adhere to CSA is lost or reduced when the *var2CSA* gene is disrupted (Viebig et al., 2005). *Var2CSA* contains multiple distinct CSA-binding domains (DBL2-X, DBL3-X, DBL5*ε* and DBL6*ε*) (Avril et al., 2006) and the exact residues involved has been mapped in two separately published crystal structures. DBL3x from the A4 parasite was over-expressed as inclusion bodies, refolded and co-crystallized with CSA. Again the tertiary structure remained very similar to the EBA-175 F1/F2 and Pkα-DBL. The protein crystallized as a monomer and binding to CSA was observed on the aft surface of the molecule, on the face that corresponds to F1/F2: α-2,3-sialyllactose binding in EBA-175 but separated from the regions previously described in DBL binding (Singh et al., 2008). This binding site was predicted from the structure of Higgins (2008), albeit no co-crystallization was performed in this study.

1.5.3.4 DBL1α and rosetting associated ligands

Most of the PfEMP1 domains mediating the rosetting phenotype have been mapped to the DBL1α head structure of the protein, although immunoglobulin-binding, which has also been found important for rosetting, has been found to be mediated amongst others by the CIDR1α- domain. Two distinct types of DBL1α domain have been described (α and α1), which differ in the number of conserved cysteines and other hydrophobic residues (Robinson et al., 2003). The domain also contains hyper conserved sequence motifs that appear in all DBL1 variants. Taylor and coworkers devised in 2000 a PCR primer set that could in theory amplify the whole DBL1α repertoire in a given sample (Taylor et al., 2000a). Bull and coworkers (2005) applied these and identified specific sequence signatures (called PolV groups) in the DBL1α domain. One of the characteristic features of PolV group 1–3 (DBL1α1) is the presence of only one or two cysteine residues in the DBL1α region analyzed, in relation to three to five residues in PolV groups 4–6. Analysis of the genome repertoire of DBL1α sequences from a global collection of isolates using group A-specific primers showed that nearly all group A DBL1α1 sequences have a two cysteine signature, i.e. belong to PolV groups 1–3 (Trimnell et al., 2006). DBL1α contains clusters of glycosaminoglycan (GAG) binding motifs which are believed to play a part in the receptor-ligand interactions (Chen et al., 1998). The highly rosetting FCR3S1.2 clone express a group B *var* gene that binds to multiple receptors (Chen et al., 2000). The binding of heparan sulfate GAGs as well as adhesion affinity to blood group A antigen has been mapped to its DBL1α domain,

albeit the exact residues involved in the interaction has not been elucidated (Barragan et al., 2000a; Barragan et al., 2000b; Chen et al., 2000; Vogt et al., 2003). The group A *var* gene expressed by the rosetting clone R29 binds to CR1, also through its DBL1α domain (Rowe et al., 1997). The receptor-binding residues of R29-DBL1α map to the central region of R29-DBLa that is equivalent to the central approximately 170-amino acid stretch spanning cysteines 5 to 8 (Mayor et al., 2005). Finally a third PfEMP1 has been characterized as a rosetting phenotype mediator called the *varO* gene (Fandeur et al., 1995; Vigan-Womas et al., 2008). *The* varO parasite is an antigenic variant of the *P. falciparum* Palo Alto 89F5 clone that was isolated from Saimiri monkeys. The *varO* is a Group A *var* gene and has high sequence similarity to *R29var1* but does not bind to CR1 nor HS. The varO-DBL1α receptor still remains elusive.

1.5.3.5 PfEMP1:Immunoglobulins

Immunoglobulin binding has been reported to involve several DBL domains and the combined data presents a somewhat confusing picture. The binding site of IgM on the PfEMP1–FCR3S1.2 adhesin has been mapped to its CIDR1α domain (Chen et al., 2000) and analysis of the PfEMP1-TM284S2 adhesin identified the DBL2β domain as the IgG binding domain. The recombinant DBL2β domain, which bound the Fab' and the Fc fragments, inhibited adherence to placenta, suggesting binding to the placental Fc receptor (Flick et al., 2001). Further, flow cytometry assays have located IgM and IgG binding in the var2CSA PfEMP1 protein of the 3D7 genome parasite. Three domains, DBL2-X, DBL5-ε, and DBL6-ε, bound human IgM, whereas IgG binding was observed only with DBL2-X and DBL6-ε (Rasti et al., 2006). In FCR3, the var1CSA and var2CSA PfEMP1 species bound IgM to DBLε domains, DBL7ε and DBL6 ε respectively (Semblat et al., 2006).

1.5.4 *var* gene transcription-expression and disease

Since switching of *var* expression occurs with a high frequency (Roberts et al., 1992), the infecting population in malaria patients is heterogeneous with regard to its surface serotype, adhesion type and expressed *var* gene. Not only is the circulating parasite pool heterogeneous in its committed *var* expression profile (Lavstsen et al., 2005), but that is also the case in specific tissues, with multiple *var* genes being expressed by the sequestered population (Montgomery et al., 2007). A noticeable exception is the placenta, where *var2CSA* transcription is univocally dominant. PfEMP1 expression has in laboratory strains shown to be developmentally regulated with an emergence of protein on the IE surface at 16-18 hours post invasion. This coincides with the adhesive phenotypes associated to disease (Gardner et al., 1996; Kriek et al., 2003). The strains used for the investigation of the timing of *var* gene transcription have typically been clonal parasites grown for very long periods of time *in vitro* prior to the experiments. These were usually refined for a specific phenotype, in the case of the above studies the parasite A4, which expresses a PfEMP1 that binds to CD36 and ICAM-1. Studies on the mRNA level, conducted with northern blots in the same A4 parasite strain disclosed that the major *var* mRNA transcript encoding the PfEMP1 variant destined for the IE surface is present at its highest level in mid-ring stage parasites. The transcript then declines to subsequently recede to barely recognizable levels in pigmented trophozoites. This suggests that the time lag between the relative peak in transcription and PfEMP1 protein on the IE surface relates to a temporal

necessity for post transcriptional modifications and transport through the IE. Studies conducted through reverse transcription PCR has presented a somewhat different picture. These indicate that many *var* genes are transcribed in ring stage parasites but only one major transcript is present in pigmented trophozoites (Chen et al., 1998; Scherf et al., 1998). These studies were conducted with sub-clones of the FRC3 parasite with respective binding phenotypes to HS, CD36, ICAM-1 and CSA. This implies activation of a silencing mechanism in the later phases of ring stage development. Abortive or incomplete transcription has been hypothesized as a possible molecular control mechanism. Numerous ring-stage transcripts are not detected by Northern blot analysis, although one study has reported multiple spliced var transcript types in pigmented trophozoites. One answer to this discrepancy could be that the RT-PCR technique because of its sensitivity picks up background noise either present as slightly inhomogeneous parasite populations or transcripts present at extremely low levels. Northern blots on the other hand lack the capability to identify low level transcripts.

Several recent studies have investigated the *var* expression profiles in patients with severe or uncomplicated malaria. The first study to observe qualitative differences in *var* gene transcripts from severe malaria field isolates compared to mild ones was performed in Brazil (Kirchgatter et al., 2002). Here the investigators found that patients suffering from multi-organ failure severe malaria predominantly carried circulating parasites that transcribed *var* genes with few cysteines in a designated stretch of the DBL1α domain. An immuno-pulldown assay of 3D7 parasites expressing commonly recognized antigens indicated that children with severe disease transcribe group A *var* genes (Jensen et al., 2004). Quantization by real-time PCR of the different 5´-upstream groups in a mixed population of *var* genes was performed in a study from Papua-New Guniea. Full length *var* gene transcripts were extracted from patients with severe malaria, uncomplicated malaria as well as asymptomatic cases. The genomic distribution of the three *var* sub-groups was similar in the three clinical groups. No statistical difference in *var* gene group transcription between the different patient categories was observed; however group A *var* genes predominated in mRNA from rosetting parasite isolates (Kaestli et al., 2006). Further evidence of an association of rosetting with such group A DBL1α1 sequences derives from a study in Kilifi, Kenya (Bull et al., 2005), our study in Uganda (Papers I) and a study from Mali (Kyriacou et al., 2006). The last study indicates that transcription of DBL1α1-like sequences is positively correlated with rosetting frequency. These field studies correspond in part to the *in vitro* data mentioned above; the rosetting parasites R29 and varO both express group A *var* genes. However *FCR3S1.2var1* is a group B gene and other *var* genes that have been implicated in rosetting, *IT4var1* and *IT4Var27* from the IT4 genome, are from group C and B respectively (Kraemer et al., 2007).

Usually a large range of transcribed *var* genes is detected in each patient sample which adds a second layer of complexity to *var* gene analysis in patient material (Bull et al., 2005; Kaestli et al., 2006; Kyriacou et al., 2006; Montgomery et al., 2007) (Paper I). Add to this the fact that multiple parasite genotypes can infect the same patient. Autopsy studies in four patients from Malawi revealed that up to 102 different *var* genes can be simultaneously transcribed in a single host and up to 14 different *var* sequences found in a single organ (Montgomery et al., 2007). The patients were infected with up to seven different *P.falciparum* genotypes. The same study also showed that parasites sequestered in different organs displayed a

differential *var* gene transcription profile. This is consistent with the hypothesis that *P. falciparum* parasites express PfEMP1 molecules that are organ-specific. It also indicates that there is a discrepancy in the PfEMP1 phenotypes in circulating versus sequestered parasites. Interestingly none of the major transcripts found in any organ were of the DBLα1 sequence group except in the spleen of one patient. Unfortunately the circulating parasites were not investigated in this study.

Another daunting obstacle that faces these types of association studies is that the extent of sequence heterogeneity in different geographical regions is not fully understood. A recent extensive study charted the global genomic variation in the same DBL1α domain, which was found to be virtually endless (Barry et al., 2007), but the PCR primers used were the same as in all previous studies made in this respect (Taylor et al., 2000a). Sequence tags from DBL1α domains that do not anneal to the degenerated primers will be missed.

1.5.5 Molecular basis of *var* gene regulation

It was devised early on that each singular parasite expresses only one PfEMP1 type on the IE surface (Scherf et al., 1998). This phenomenon is referred to as mutually exclusive expression and recent studies have revealed that it is transcriptionally regulated and independent of antigen production (Dzikowski et al., 2006; Voss et al., 2006). These data implies that changes in *var* gene transcription are not activated by external stimuli, thus the parasite regulates transcription itself to create switch variants. Transcriptional switching need to occur at a high enough rate to generate parasite subpopulations that escape the human immune response yet be tightly controlled in order to avoid premature expenditure of the genomic *var* gene repertoire. An early study demonstrated that parasites cultured *in vitro* devoid of immune pressure switch spontaneously at a rate of 2% each generation (Roberts et al., 1992).Whether the switching is a random process or not is under debate. One study using the A4 parasite indicates that no preferential switching occurs between central or telomeric *var* genes (Horrocks et al., 2004). Another study gives evidence to that central *var* genes switch more frequently than subtelomeric ones (Frank et al., 2007), in this case the lab clone NF54 was used. These two lab strains have been proven to have different switching rates *in vitro,* an observation that may explain the discrepancy. Several lines of evidence suggest that regulation of *var* gene transcription is a multi-layered system (Horrocks et al., 2004). The first layer includes the two promoters found in virtually all *var* genes; the 5´upstream region and the intron. Several studies investigating *var* promoter function in transiently or stably transfected parasites suggest that *upsC* promoters are activated by default and only silenced in the presence of a *cis*-linked *var* intron(Deitsch et al., 2001; Gannoun-Zaki et al., 2005; Frank et al., 2006). Thus, these genetic elements confer the ability to silence each individual *var* gene as an epigenetic unit. Recently a second layer of regulation has emerged consisting of chromatin modifications, specifically histone deacytelation (Freitas-Junior et al., 2005) and methylation (Chookajorn et al., 2007; Lopez-Rubio et al., 2007). The first study used a chromatin immunoprecipitation assay to show that the upstream regions of active *var* genes are associated with acetylation of histone H4 while the silenced *var* genes are flanked upstream by hypoacetylated histones. The same method was used in the other studies to illustrate that trimethylated histone H3 at lysine-9 (H3K9me3) is significantly enriched at silent *var* genes and H3K4 tri- and dimethylation in the 5´flanking regions

classifies active *var* genes. Different epigenetic factors appear to be involved in the regulation of different *var* gene groups. The yeast SIR2 protein (silent information regulator 2) plays a central role in silencing by marking chromatin through deacetylation of histone tails. The *P. falciparum* genome encodes a SIR2 homolog (PfSir2) who seems to function as a silencer of certain *var* gene subsets. In a micro-array based study, the *var2csa* and *upsA var* genes were de-silenced in PfSIR2 knock-out parasites, whereas transcription of *upsB* and *upsC var* genes was largely unaffected (Duraisingh et al., 2005).

1.6 MALARIA GENOMICS

The *Plasmodium* nuclear genomes (Carlton et al., 2002; Gardner et al., 2002; Hall et al., 2005; Carlton et al., 2008; Pain et al., 2008) are estimated to contain 23–27 million bases, 14 chromosomes and about 5500 genes. They are rich in low-complexity regions and most have a high A+T content in comparison to other protozoans. The genome of *P. falciparum* is exceptionally (A+T)-rich, with a whooping 79.6% A+T content throughout the genome. Circa 60% of the genes in *P. falciparum* lack any sequence similarity to genes from any other sequenced organism, which poses a great challenge in identifying the genes for drug resistance and vaccine targets in the genome. Detection of over 2400 proteins by mass spectrometry showed that a large number of these hypothetical open reading frames are transcribed (Florens et al., 2002). The genes with a known function have an overrepresentation of genes involved in immune evasion and host-parasite interactions. Underrepresented are genes associated with cell cycle, organization, biogenesis, enzymes, transporters and transcription factors (Gardner et al., 2002). This disproportion reflects maybe more our lack of understanding of basic cellular processes in the parasite rather than an actual skewedness in distribution. There are vastly different rates of variability in different parasite gene classes, with many genes involved in 'housekeeping' functions, such as ribosomal proteins, DNA replication enzymes, or components of the cytoskeleton, exhibiting very low levels of variability as one compares the genomes of different *P. falciparum* isolates from different continents. This implies that the species has travelled through a bottleneck in the recent evolutionary past. In contrast the rate of variability is disproportionally high in the telomeric chromosome ends and the reasons for this have already been extensively discussed (Volkman et al., 2002). Predicted surface proteins have in general a high rate of genotype divergence even if they are not predicted to be a member of any VSA family. Examples are the proteins chosen as vaccine candidates, the CSP, AMA-1 and MSP proteins which may explain the allele-specific immunity that is sometimes observed (Polley et al., 2007).

Genetic diversity in *Plasmodium* have been studied in various ways, from the beginning with the serial analysis of gene expression (SAGE) methodology that sequenced short regions of transcribed genes. The premier finding in this work was that a large part of the sequence tags (17%) corresponded to the antisense strand of annotated genes (Patankar et al., 2001; Gunasekera et al., 2004) This was speculated to play a role in transcriptional regulation, but no definitive evidence to support this hypothesis has appeared. The genome sequences provided researches with opportunities to map both expression and genomic variation though microarray hybridizations. Two major studies on global gene expression over the whole lifecycle have been conducted (Bozdech et al., 2003a; Le Roch et al., 2003). Despite different

experimental setups, 25-mer oligonucleotides (Le Roch et al., 2002; Le Roch et al., 2003) versus 70-mer dittos (Bozdech et al., 2003a; Bozdech et al., 2003b), both studies showed comparable patterns of expression in the IE stages. A number of comparative genomic hybridizations (CGH) studies have been done on *P. falciparum* parasites; (Bozdech et al., 2003a) mainly for exploring the suitability of oligonucleotides to be used in transcriptional analysis and (Carret et al., 2005) investigating the suitability of whole genome amplifications (WGA) on Affymetrix microarrays. Furthermore (Kidgell et al., 2006) investigated genetic variation in *P. falciparum*, as well as we did with our 70-mer CGH (Paper II). These studies have identified a number of gene that are implicated in gene amplification and deletion events, which among others has relevance to drug resistance, i*n vitro* growth and host-cell interactions. This year the overall variability in five strains was mapped with a 2,56 million probe tiling array, a resource that could also penetrate the subtelomeric compartments (Jiang et al., 2008). This was previously impossible because of the extreme sequence redundancy in the regions. Furthermore full genomic diversity maps have recently been established by several groups (Jeffares et al., 2007; Volkman et al., 2007), this has further widened the grasp of the parasites´ evolutionary ability to develop drug resistance, evade immunity and establish chronic infections. Lately with the advent of ultra-mass sequencing, for example with the 454 or SOLID methodologies, new venues have been opened to explore the full extent of the malaria genomic variorum.

2 SCOPE OF THE THESIS

The aim of this thesis was to further the understanding of how surface antigens contributes to severe disease in *Plasmodium falciparum* malaria and if there are specific genetic hallmarks to severe disease.

SPECIFIC AIMS

The specific objectives of the different papers were as follows

Paper I

Characterize *var* gene sequences of parasites from patients suffering from severe malaria in relation to parasites of patients with uncomplicated disease.

Paper II

Investigate the global distribution of *P. falciparum* gene deletions and duplications in both laboratory and field isolates.

Paper III

Determine *var* gene transcription patterns in *P. falciparum* field isolates.

Paper IV

Investigate the *in vitro* proliferative capability of *P. falciparum* from patients with different disease states.

3 EXPERIMENTAL PROCEDURES

A number of new methods had to be established in the line of the work with this thesis. This chapter will focus on the methodological considerations, strengths and weaknesses associated with the different methods. A more detailed description of the respective assay can be found in the appropriate paper.

3.1 MODIFIED PARASITE CULTURE THROUGH ORBITAL AGITATION AND GAS

The method of how to culture parasites continuously *in vitro* was established in the seventies (Trager et al., 1976) and the methodology has changed little since. The candle-jar technique is basically an assay where infected erythrocytes are placed in a 5% hematocrit solution with supplemented malaria cultute medium (MCM). In addition either 10% human serum or Albumax is added. The lid to the culture flask is opened; the flask placed in an air tight jar and the oxygen is burned away with a candle. Most lab strains and *in vitro* adapted isolates have been cultured this way. Malaria investigators have historically had difficulties in *in vitro* adapting field isolates, with poor outgrowth in many trials. We built on a protocol from Butcher and coworkers 1981. The aim was to try to mimic the more turbid environment in the circulation as well as to try to optimize the nutritional exchange in the culture. The liquid medium prerequisites are the same but the air in the culture flask is replaced by a gas mixture of 5% CO_2 and 5% O_2 in nitrogen. The culture flasks are placed in an orbital shaker with 50 revolutions per minute and growth is continuously monitored. As shown in paper IV, the outgrowth of the *in vitro* adapted strains were significantly improved and continuous cultures of field isolates could be established in all cases.

3.2 DBL1ALPHA RT-PCR AMPLIFICATION

The degenerated PCR primer set (α-AF/ α-BR) constructed by Taylor and coworkers 2000 were designed to recognize all DBL1α sequences in a non biased way. We found however empirically that the primers have a bias toward a subset of sequences, for example the varCOMMON *var* gene that had implications on our semi-quantitative approach of elucidating *var* gene dominance in a heterogeneous mix of *var* gene transcripts. We designed a pair of complimentary PCR primers (nDBLf/nDBLr) as a way to circumvent this and we could see that the overall coverage of the 3D7 var gene repertoire was also improved. These primers map to different semi-conserved regions in the DBL1α domain than the α-AF/ α-BR primers and we used them in conjunction to and in combination with (α-AF/nDBLr) the original primer set. The PCR conditions were the same in all the amplifications. This supplied us with a less biased dominance order on which we could build our analysis of the dominant *var* genes in the different field isolates.

3.3 MASSIVE DBL1ALPHA FRAGMENT SEQUENCING

In order to ascertain a dominance order in the *var* genes amplified in the semi-quantitative PCRs we resorted to a mass sequence methodology previously used in whole genome sequencing projects. We used the Mega-Bace machinery and reagents supplied by the manufacturer, in clone-by clone sequencing. 48 PCR inserts from each PRC product were cloned using the TOPO TA cloning kit (Invitrogen, Carlsbad,

CA) and cycle sequenced both in the forward and reverse direction. Sequence reads were base-called using phred version 020425.c, vector screened using cross_match and quality trimmed, in the same way as gDNA library reads are handled. Since the primer regions are conserved and primer mismatch may have occurred in the PCR amplification, the quality values of the primer region in each read were artificially reset to 0 before assembly. The reads were clustered using phrap ([Green et al, unpublished, http://www.phrap.org] version 0.990319), with retain_duplicates, minmatch 20 and repeat_stringency 0.9 and otherwise default settings. The lowering of the repeat stringency was implemented because of a tendency of phrap to cluster the same DBL1α sequence in different consensus sequences (contigs) depending on a single nucleotide mismatch. Since maintenance of *var* gene heterogeneity relies on larger recombination events we reasoned that these SNPs were the result of sequencing or Taq enzyme error. We tested different stringencies and found that a slight lowering eliminated the majority of these erroneously sorted contigs. The translations of cluster consensus sequences were scanned for the presence of the RSFADIGDI motif using fuzzpro, (EMBOSS 3.0.0) with 3 mismatches allowed. Stop codon free reading frames containing the motif were retained. Furthermore the AmpliTaq enzyme has difficulties counting multiple nucleotide residues in long mononucleotide repeat stretches. Therefore a realignment, an implementation part of the GRAT package was made and new consensus sequences were called. This is in essence a routine that scans the contig for non agreement positions in the read consensus and does a majority vote. Reading frames with the RSFADIGDI motif with at most 3 mismatches and fewer stop codons than for the corresponding un-realigned cluster were retained. The translation ensured with automaticity that non-DBL1α sequences such as DBL2β and spurious human and bacterial sequences were eliminated.

3.4 THE MOTIFF *VAR* GENE MOTIF FINDER

Our method to identify structural DDL1α sequence tags overrepresented in different malaria disease states is based on a sub-sectioning of multiple alignments and sorting of the short sequences in biochemically similar consensus motifs. The in-data consists of a protein alignment and dominance count data. We chose the BLOSSUM62 protein alignment matrix since it aligns the amino acids sequences primarily according to biochemical likeness. MOTIFF functions so that that the alignment is divided according to homology into columns of high medium and low identity. Ultra conserved, un-gapped regions of the alignment are primarily identified (as a consecutive stretch of alignment columns with over 90% identity) and for each such region a representative seed sequence is chosen. PSIBLAST (Altschul et al., 1997) (NCBI blastpgp v2.2.10) with the BLOSUM62 substitution matrix, multipass inclusion threshold 10, expectation value 100 and hit extension threshold 4 is subsequently used to iteratively identify other PfEMP1 sequence members containing that motif. We decided to include two analysis settings in the motif identifier, either a strict or a non-strict analysis. In the case of the non-strict setting the motifs can be constructed from any previously un-hit part of the aligned sequences. The strict option searches only the homology column from which the seed sequence is found, and a small window surrounding them. The strict setting was applied in our analysis. This generates strictly localised motifs, and the small window allows correct detection even with alignment inaccuracy. The remaining un-hit and unused sub-sequences in the

ultra-conserved columns are subsequently tried as seed sequences. Iterative PSIBLAST searches with the same parameters are made until no un-hit or unused sequences remained in the selected columns. When this is completed, the search routine continues to pick out seed sequences from the medium homology column. The same stepwise PSIBLAST approach is applied to consecutive stretches with identity above 45% until this column is also depleted and so on until nothing in the alignment remains to be seeded or searched. In the third step, high identity islands in columns with a gap consensus residue are searched. Fourth, any columns that were still not denoted as used are seeded and searched until the whole alignment is depleted of potential seed sequences.

We wanted to be able to correlate back to the different disease states of the patients, so after this a database of position specific score matrices for all motifs was constructed. In this step the motif members are referred to the respective disease state group from which they originate and the dominance labeled member clusters are counted for each motif, effectively tallying the distribution of the motif in each patient group. Overrepresentation in dominant clusters is noted. Skew equals $(n_A/N_A - n_B/N_B) / (n_A/N_A + n_B/N_B)$ where n_A and n_B are the counts of a particular phenotype A or B in the motif, and N_A and N_B the total count of phenotype A and B respectively in the dataset. The hyper-geometrical probability P_k of finding that level of overrepresentation or more by chance in a motif k of the given size is calculated, and used to sort the motifs. The overrepresentation calculations rely on the binary relationships in each category ie either severe or mild disease, high and low rosetting rate, cerebral malaria or not and so forth.

3.5 KULLBACK-LEIBLER DISTANCES

This method has previously been used to indicate areas of relevance in degenerated sequence material (Dahlback et al., 2006). It essentially looks at each position in an alignment and notes the relative overrepresentation of specific residues in different disease groups and/or phenotypes. We used the same BLOSSUM62 matrix alignments to achieve a second layer of analysis.

The Kullback-Leibler distance between the amino acid frequency distribution in each tested phenotype dichotomy pair at each position in the alignment is calculated as $D_{KL} = \sum_i p_i \log \frac{p_i}{q_i}$, where p_i is the fraction of residue i at an alignment position in peptides from one phenotype, and q_i similarly for another phenotype.

In order to assess the significance of each distance, 10000 random phenotype assignments are drawn with the same number of labels as in the test dichotomy. A probability density function of D_{KL} is estimated for each position. Only positions with D_{KL} larger than the 95% quantile of this distribution of D_{KL} from random phenotype assignments are considered significant. The Kullback-Liebler analysis is extremely dependent on the quality of the alignment since each position of the alignment is assessed individually. The MOTIFF software is not so since a certain window is allowed in the seed-match routine. Both ways to associate between disease/phenotypes and sequence relies of course very much on which sequences are fed to the respective algorithms. Since we applied a semi-quantitative PCR we included the three most dominant sequences that appeared in each patient, while it may very well be so that only the most dominant one generates the adhesive phenotype.

3.6 ABSOLUTE QUANTIFICATION OF *VAR* GENE TRANSCRIPTS IN WILD ISOLATES

Since PCR is a stochastical process, bias will always be a factor in such an assay, we tried to further develop the assessment of *var* gene dominance in the analysis by Q-PCR. We already had the capability to sequence a large part of the *var* gene pool transcribed in the isolates. We utilized the hyper variable regions to design discriminatory Q-PCR primers to a large part of the dominant *var* genes, usually ten, but less if there were no more dominants. We took advantage of the interprimer region III (Paper I) that in most cases contain a reasonable G+C content and no long poly-A stretches which is the case of region I and region II. All amplifications were run using the Syber-Green assay system in 384 well plates in an ABI7900 Applied Biosystems cycler. The relative amplification efficiency of each primer pair was measured in standard curves from gDNA extracted from the same isolate. We aimed in the study for a concurrence of amplification efficiencies, and primer pairs that deviated more than two CT units from a common median was remade. Standardization of amplification efficiencies was carried out through a comparison to expression of the house keeping gene Seryl-t-S RNA synthetase as described in the paper. In this way we could quantify with extreme precision the relative quantities of each transcript in the pool of *var* genes in the mixture from each patient. The inherent weakness of the Syber-Green assay without using probes is that the resolution is lost at very high CT values. We had difficulties of attaining good resolution at CT´s higher than 34-35, which indicates a theoretical number of transcripts between 32 to64 copies per well. Since the amount of material from the extraction of the original trophozoites was quite low the resolution in these samples was lower than in the in vitro adapted time points where we could ascertain a good yield.

3.7 COMPARATIVE GENOMIC MICROARRAY HYBRIDIZATION

The *P. falciparum* whole genome microarray harbors a total of 6850 70-mer oligonucleotides printed in quadruplicate. Due to the fact that the microarray was originally developed for expression analysis with oligonucleotides spanning regions where introns are spliced out, and designed before the completion of the entire genome sequence of *P. falciparum*, for CGH-purposes the oligonucleotides had to be redefined based on the 3D7 genome. All oligonucleotide sequences generated based on the 3D7 strain were blasted (NCBI BLASTN 2.1.2 without low complexity filter) against the *P. falciparum* genome build from 2004/10/06 acquired from www.PlasmoDb.org. Only the ones fulfilling the criteria of: (a) generating a match to the expected sequence in the genome with an expect value $\leq 1 \times 10^{-26}$ and (b) having no other matches with expect values $\leq 1 \times 10^{-26}$, were considered to map uniquely to the genome and were included in the CNP-analysis (5985 out of 6850 oligonucleotides). The chosen e-value cut-off was empirically determined, based on for example mismatch controls, hybridization patterns of parasite DNA known to be sequence polymorphic in oligo regions as well as negative controls and strain specific oligos. Oligonucleotides not fulfilling these criteria were considered as being too prone to generate unspecific hybridizations.

Microarray data was processed using the freely available KTH-package (Wirta et. al [http://www.biotech.kth.se/molbio/microarray/pages/kthpackagetransfer.html]), in the R environment for statistical computing (http://www.r-project.org/). Flagged features and features with median signal intensities lower than two times that of the background in both channels were considered being of bad quality and filtered out. The

data without any background subtraction was normalized using a print-tip dependent LOWESS approach with $f = 0.5$ (Yang et al., 2002). No between-slides normalization of achieved ratio values was considered necessary. In order to rely on a more robust method than the fold-change approach to identify oligonucleotides displaying differential hybridization, we took advantage of the fact that all the oligonucleotides were printed in quadruplicates. B-statistics (Smyth 2004) performed with within-slide replicates weighted down in importance compared to between-slide replicates, together with Bonferroni's adjustment for multiple comparisons were therefore used. Array elements achieving p-values ≤ 0.001 were considered to be deviant and statistically significant. The selected p-value acts as cut off for the microarray data in all plots and tables shown in this work. Indirect comparisons of all strains and isolates (based on their ratio differences to 3D7AH1) were executed in the same way. In addition to above stated processes, array elements displaying potential dye bias based on the performed comparative and self–self hybridizations were considered uncertain and filtered out.

3.8 FLUORESCENT IN SITU HYBRIDIZATION (FISH)

The FISH was conducted according to previously described methodology (Freitas-Junior et al., 2000; Ralph et al., 2005), with some modifications. The dsDNA probes were amplified using specific primers with a high TM in stringent conditions. Both probes were designed towards highly conserved regions, with minimal risk of cross hybridization to other genes. The probes were labeled using Fluorescein-High Prime kit (Roche Applied Science) and purified using the MinElute PCR purification kit (Qiagen). The parasites were isolated from their host erythrocytes using saponin (0.05%, w/v) and carefully smeared as monolayers on regular microscope slides. The parasite preparations were air-dried and subsequently fixed with 4% paraformaldehyde (PFA) for 15 min at room temperature followed by washing in PBS. Without letting the monolayers dry, they were immediately treated with RNase (20 μg/ml in 2× SSC) for 30 min at 37 °C both for the sake of permeabilization and decrease of background. This proved to be an important step in avoiding craquelation of the nuclei. Preparations were washed again in PBS and then pre-hybridized under a sealed rubber frame in heat denatured (95 °C for 3 min) hybridization solution (50% deionized formamide, 10% dextran sulphate, 1× SSC and 250 μg/ml herring sperm DNA) lacking probe at 42 °C for 30 min. The hybridization solution was removed and exchanged for new containing 100 ng of labeled and heat denatured probe before hybridization at 95 °C for 3 min and at 37 °C for 15 h. After hybridization, parasites were washed twice in 50% deionized formamide/2× SSC at 50 °C, twice in 2× SSC first at 50 °C then 55 °C and once in 4× SSC at room temperature. Parasites were finally washed twice in a solution of 100 mM Tris–HCl/150 mM NaCl/0.5% (v/v) Tween 20 at room temperature and mounted in Vectashield (Vector Laboratories). Preparations were visualized using a Leica DMRE microscope and imaged with a Hamamatsu C4880 cooled CCD camera.

4 ETHICAL CONSIDERATIONS

Ethical approval for the human component of the studies conducted in this dissertation was obtained from the research ethical committee in Uganda as well as Karolinska Institutet. Informed consent was obtained from the parents or legal guardians of the patients. Approval for the experiments involving antisera from animals was obtained from the animal ethics committee at Karolinska Institutet.

5 RESULTS AND DISCUSSION

5.1 PAPER I

In order to explore the relationships of *var* gene transcription in malaria field isolates we had to work in a suitable location. Two areas were chosen for their distinct panorama of malaria endemicity. The first group of patients was recruited at the district hospital in Apac, Uganda, which serves a rural community of about 200,000 individuals; furthermore an unknown number of refugees from the northern part of the country were served during the study period. The malaria transmission is pan-seasonal and the rate of infective bites per night is the highest recorded in the world to date (1563/year (Yeka et al., 2005). This extremely high inoculation rate is partially attributed to poverty, political unrest, and the vast marsh lands surrounding the city. The medical officer in charge screened the patients for signs of malaria upon admission and a blood smear was taken. The patient's blood- haemoglobin and glucose levels were measured. The patients in the severe malaria category were all admitted to the ward and treated lege artis. The patients in the mild malaria category were treated ambulatory and did not stay for care. Thirty-eight patients (n=38) were included in the severe malaria group and forty-four (n=44) patients were included in the mild malaria group. The mean age of the children were 1.3 years (1.23 , severe; 1.4 mild). Secondly a number of patients from Mulago hospital in Kampala were included in the study. Mulago hospital is situated in the capital and serves as a referral hospital for the whole southern region of Uganda. The malaria transmission varies in the region with a holoendemic situation to the north and east and lower transmission (mesoendemic) to the west of the city. The patients were screened as before by the physician in charge of the ER. The patient's blood Haemoglobin and blood Glucose levels were measured with Haemoque machines. Fourteen patients (n=14) with severe malaria were included in the study. The patients were all admitted to the ward and treated. The mean age of the children was 2.2 years. The median ages of the patients reflect very well the endemic situation and the disease panorama as well. The severe malaria patients in Apac suffered mainly from respiratory distress syndrome and the patients in Mulago had an overrepresentation of cerebral malaria cases.

Blood samples were drawn and the red blood cells (RBC) separated from other blood constituents and the parasites cultured for up to 35h until the majority of the cells were matured into trophozoite stage. The reason behind this somewhat complicated procedure are the data indicating that trophozoite stages display the adhesive phenotypes associated to disease and that *var* gene transcription in early stages have been shown to be pluripotent (see above). Parasite phenotypes such as rosetting rate and the presence of giant rosettes were noted. Trophozoites were enriched and then extracted for total RNA, precipitated in isopropanol, stored in -70°C and transported to Sweden for further analysis. The RNA was then reverse transcribed and three separate PCRs were run with the degenerated DBL1α primer sets described above. The amplicons were sequenced and the reads assembled and the resultant contigs were run through the MOTIFF script pipeline and correlated to the different phenotype and disease state categories.

We found that we could identify a number of degenerated motifs statistically overrepresented in each category, in all 6 motifs related either to high rosetting rate and severe malaria in general (3 in each respective category) and 12 motifs if the severe malaria patients were subdivided into sub-categories, cerebral malaria, severe malaria NUD and respiratory distress syndrome (2, 4 and 6 motifs respectively). One has to remember that the groups were compared to its counterpart ie severe malaria-mild malaria, cerebral malaria-non cerebral malaria and so forth. There was certain overlap among the motifs for example the low rosetting motif L4 is also a member in the mild motif category termed as M4 and the severe motif S1 is also represented as a motif overrepresented in the respiratory distress category as R3. The Kullback-Liebler plot analysis coincided in location of the statistically overrepresented residues in each category in greet parts with the location of the motifs, however there was not total coherency. To further the analysis we wanted also to try to map the motifs in a structural context. All the DBL domains structurally determined to date display a similar base configuration with a conserved scaffold of alpha helices surrounded by flexible loops. We constructed therefore models of characterized DBL1α domains using the EBA-175 F2 domains as a template. The EBA-175 F2 has the highest sequence similarity to DBL1α and was predicted to yield the most accurate model. Motifs present in the respective domain (FCR3S1.2var1-DBL1α, R29var1-DBL1α1 and VarO-DBL1α1) were located in the model structures and found to coincide with the binding faces of EBA-175 (FCR3S1.2var1) and Pkα-DBL (R29var1-DBL1α1 and VarO-DBL1α1). This finding prompted us to study the immune recognition of the Ugandan isolates by using two different sera raised to DBL1α of FCRS1.2. We found that the mean serum reactivity to the IE surface was higher with the isolates in the severe relative to mild category.

Our hypothesis is that these motif sequences in the DBL1α have an importance in binding efficiency to various host receptors. The parasite has to vary the sequences of the VSA domains but has to retain function. We found that the motifs typically located to the putative alpha helical semi-variable sequences that flank the hyper-conserved domains, which is logical in a sense. The hyper-variable predicted loop regions are unlikely to contribute significantly to binding because of their inherent redundancy, however the length of these regions could be of import. It has been proposed that the loop regions function as flexible decoy or lid that shield the binding residues from antibody recognition (Higgins 2008). The conserved regions are predicted to locate to the interior scaffold of the molecule and should not be either directly involved in receptor binding events nor be exposed to the immune system. The motifs are in our eyes potential candidates for the construction of blocking antibodies and work is in progress to create such. The extent of cross-reactivity of αDBL1α-motif antibodies remains to be seen.

5.2 PAPER II

Gene duplications and deletions are important features in the creation of genetic diversity within a species. We wanted to understand how frequently copy number polymorphisms occur in the genome of the malaria parasite and what impact they have on parasite traits. We did this by measuring relative DNA abundance levels in a number of *P. falciparum* strains and isolates of different geographical origins. Two isolates from Apac were included in the study together with 8 strains selected because of phenotypic differences in growth, rosetting, cytoadherence, the presence of knobs, the capacity to form sexual stage parasites and differences in the sensitivity to antimalarials. We used a 70-mer microarray platform with oligonucleotides printed in quadruplicates covering nearly the whole genome, and conducted dye-swap comparative genomic hybridizations against 3D7 reference strain DNA. Hierarchical clustering of statistically significant $\log_2$ ratio differences revealed relatedness in between strains and a surprisingly high resemblance was found between three of the lab strains.

The same data set was used to detect copy number polymorphisms (CNP). VSAs and all the highly variable subtelomeric regions were separated out by guilt of association from the more homogenous centromeric regions and omitted from the analysis. This was done to avoid mistaking sequence polymorphisms as duplications and deletions. Significant signals from at least two oligonucleotides, consecutively mapped to the genome, were required for CNP calls. 24 CNPs involving 82 genes were recognized in this way, 50 genes with increased amounts of copies and the rest with decreased relative copy numbers. The size of the CNPs varied between 1 to 110 kb and ranged from being constituted of 1 to 22 genes.

Validation of the CGH results was performed by fluorescent in situ DNA hybridization and quantitative PCR on a subset of the CNPs. Amplifications of genes encoding SURFIN4.1 and a reticulocyte binding like protein (PfRh1) had the highest test strain over reference ratio, corresponding to a copy number difference of six. These amplifications were found in two of the strains that had the highest in vitro multiplication rate, possibly suggesting amplifications of invasion genes important for efficient parasite multiplication. FISH was used to confirm the amplifications as well as localizing the additional gene copies to different chromosomal ends. The most extensive CNP, both in terms of size and frequency, was located on chromosome 5, included 22 genes and displayed a cumulative size of ≈110 kb (the size of overlapping regions in different strains). Six out of ten parasites displayed clear copy number gains in this region, also confirmed by real-time QPCR. The duplications varied in size from ≈27 to 93 kb and spanned different genes in the different parasites. Two particular genes, PFE1150w (*pfmdr1*) and PFE1145w, were amplified in all strains, potentially identifying hot spots for gene duplications on chromosome 5. The pfmdr1-gene has previously been found to be prone to copy number gains both in vivo and in vitro, rendering the parasite resistant to mefloquine and quinine (Cowman et al., 1994; Price et al., 2004). The parasites lacking these duplications were all sensitive to quinine while most that carried multiple pfmdr1 copies were found resistant to mefloquine and to quinine. In addition to the described examples of genes involved in red blood cell invasion and drug resistance, putative genes associated to cell-cycle regulation and meosis were found varying in relative copy numbers.

Considering the size and numbers of CNPs found in the limited number *P. falciparum* strains covered in this study, it is unlikely that these events are selectively neutral. The nature of genes covered by CNPs, suggests that the malaria parasite employs gene duplications and deletions as a general strategy to enhance its chances of survival and spread. We could both find new and confirm previous CNPs in a wide range of parasites, some with shown correlation to function and parasite phenotype. Future work includes a more extensive selection of wild isolate strains to appreciate the wider distribution of CNPs in the wild type situation

5.3 PAPER III

As mentioned previously the analysis in paper I was built up on the premise that the three most dominant DBL1α domains amplified from each isolate contributes to disease. This is an arbitrary assessment and we proceeded to try to further assess the importance of the constituent DBL sequence fragments in paper III. We also intended to explore the transcriptional dynamics of individual *var* genes in the wild isolates in a developmental context. In order to do this we chose 14 Ugandan field isolates, 11 samples from patient with severe malaria and 3 samples from the mild category. The samples originated both from Apac and Kampala. Seven clonal lab strains with different phenotypes were also used in the assay. We began by performing three separate semi quantitative PCR amplifications on cDNA from all the parasite samples in both early (6-10h) and mid-trophozoite stage (22-26h post invasion). The amplicons were sequenced according to the same procedure as in Paper I. The sequence reads were assembled and dominance list of consensus reads could be established for each isolate and time-point. In the case of nine field isolates we used 0 generation parasites, ie parasites that had not been *in vitro* adapted but only directly processed or allowed to mature to tropozoites, as well as the 7 lab strains. Five isolates were *in vitro* adapted and studied as ring stage or mid stage trophozoites after 6-11 generations *in vitro*. Moreover, a time course of *var* transcription of five time points over the life-cycle was established for the isolate UAS31. To be able to study in detail the different relative quantities of each *var* gene in the mix of transcripts in the *in vitro* adapted time-points we designed discriminatory Q-PCR primers to the ten most dominant *var* genes in each sample. We also designed primers to target the unique semi conseved var genes varCOMMON, var2CSA and var3, and put them in relation to the genes unique to each isolate. Quantization through Q-PCR was also conducted in two of the lab strains. The Q-PCR assay proved to have very good resolution, but the concentration of cDNA had to be relatively high in the samples. The 0 generation samples contained too low concentrations of cDNA to be assessed in this way.

We found in the semi-quantitative PCR assays that the order of dominance of each var gene fluctuated between the two developmental stages in all isolates tested. Albeit in most cases there was coherency with at least one of the three dominant genes being present in both stages. We reasoned that this could maybe be an effect of primer bias or an actual flux in relative levels of transcripts as the parasites mature. The Q-PCR quantification trials revealed a fairly complex picture with no absolute trend. We found that the *var* gene transcriptional profile of each isolate is unique to each isolate/clonal strain. The peak in var gene transcription in relation to time point seems to vary among samples. In four of the samples, peak transcription occurs in the mid trophozoite time point. One isolate displayed roughly equal levels in the two developmental stages. Two samples showed peak transcription in the early ring time-point. One isolate also reordered primary dominants in early versus late stage development. There was an association between the dominant genes found in the semi-quantitative PCR and the Q-PCR, but never an exact co-ordering in the two assays. We found furthermore that to ascertain the correct dominance all three primer pairs had to be taken into consideration.

Key publications investigating *var* gene transcription over time have mainly been conducted on laboratory strains that have been cultured for many years and generations *in vitro*, actually mostly in the FCR3 parasite (see above). In the case of field studies,

there is no consensus on how they should be conducted. In many previous studies investigators have extracted RNA from ring stage parasites and it is then assumed that there is no change in the dominant *var* gene between the loose transcription in early ring stage and the more controlled transcription in later trophozoite stage. Only in a few studies have one used trophozoites directly from tissues such as placenta or different organs at autopsy or cultivated the fresh parasites from ring to trophozoite stages before RNA extraction in order to avoid multiple *var* transcripts. We observed relative early switches (6 generations at the earliest), which could pose a problem for the investigator wanting to associate disease states in the patients and relative *var* gene transcription. Of course, the farther from the patient the higher the risk of antigenic drift. Since our data also show that the relative inter-isolate *var* gene transcription dominance order changes during development, the trophozoite-IE seem to be the optimal source of RNA to determine the translated *var* gene species.

5.4 PAPER IV

Most of the Ugandan field isolates were in vitro adapted at some point so as to investigate if there were inherent phenotypic traits that could be attributed to the parasites from severe or mild cases. In this work 76 isolates were studied, 36 from severe disease state patients and 40 from patients with uncomplicated malaria, a large part of the isolates were the same as in Paper I. We observed in our in vitro propagated isolate cultures a propensity of the parasites in the severe category to have a higher multiplication rate than the parasites from the uncomplicated category. This associated as well to the parasitemia of the patients at sampling, which was generally higher in the severe disease states. Another early observation was that the parasites of the severe category had a higher rosetting rate than the mild, a correlation that has been observed in many other field studies (see above). This was detected already during cultivation in Uganda, albeit the rosetting rate was significantly higher after thawing and subsequent generations in culture in Sweden. A reason to this could be that we only used one batch of human AB+ serum in the cultures in Uganda whereas during the in vitro adaptation procedure in Sweden we used a mixed pool of AB+ serum from three donors. We have seen that the extent of the rosetting phenomenon seen in parasite cultures are affected by the serum batch used (non-quantified observation). We found furthermore that all the in vitro adapted cultures formed rosettes, but to varying extents. and that rosetting persisted throughout schizogony. The rate of schizont rosetting was also higher than at mid-trophozoite stages (20-30 h), the developmental state that has previously been monitored in these types of studies. Laboratory strains previously selected for a high rosetting rate and shown to express high amounts of PfEMP1 on the IE surface (FCR3S1.2, TM284S2 etc) was parameterized in the same way. They displayed similar abilities to continuously increase rosetting through development from trophozoite to schizont, whereas long-term propagated, non-selected parasite clones or lines with little or no PfEMP1 on the IE surface did rosette, but at much lower levels.

We hypothesized that rosetting is associated to the parasites ability to reinvade new erythrocytes and that this is mediated through PfEMP1:ligand interactions on the surface of the IE, a theory put forth some time ago (Wahlgren et al., 1989). Indicative of this has been the occasional observation of several newly infected erythrocytes surrounding a bursting schizont infected erythrocyte and the presence of ring-infected erythrocytes in the microvasculature of children who succumb in cerebral malaria (Silamut et al., 1999). We recognized that we could observe the same phenomenon of newly infected erythrocytes surrounding at times an empty space, held together by what we reasoned to be the ghost remnants of an infected erythrocyte membrane. The levels of this phenomenon were observed in higher frequencies in the clinical isolates compared to lab-strains. This proximity effect was documented in a live capture microscopy film where merozoites from a bursting rosetting parasite invaded bound erythrocytes. Earlier studies have not found conclusive evidence to the association between rosetting and invasion (Clough et al., 1998; Deans et al., 2006). Our reasoning was that suboptimal methods for *in vitro* cultivation of clinical isolates had been utilized in these trials. Therefore a systematic evaluation of the in vitro adaptation procedure was conducted, and the gas-agitation method was found to be vastly superior to the static candle-jar technique in this respect. All field isolates could be established as continuously grown cultures with satisfactory parasite

outgrowth with high rates of parasite multiplication and a preserved adhesive capacity over time.

In order to investigate the role of PfEMP1 in the rosetting-invasion relationship we hindered known PfEMP1 mediated rosetting interactions with different approaches. We started by testing the FCR3S1.2 clonal lab strain by blocking the DBL1α interaction with DBL1α antibodies raised against the PfEMP1-DBL1α species known to be expressed in high amounts on the IE surface of this parasite. This abrogated FCR3S1.2 rosetting in both trophozoite and schizont stages and had a concordant negative effect on invasion. The same sera failed to disrupt schizont rosettes or affect the invasion of the isogenic, but PfEMP1 species distant, parasite clone FCR3S1.6. An absolute effect was seen on both parasite lines when a pooled hyper-immune serum from Malawi was used to disrupt rosettes. Since both IgM and IgG has been shown to act as intermediates in rosetting (see above), IG binding was blocked by adding αIgM and αIgG antibodies to the cultures. This reduced rosetting in a subset of the lab strains and in vitro propagated isolates. In the case where rosetting had been affected there was also a reduction in invasion rate with a more pronounced affect on the αIgM treated parasites in general. Heparan sulfate-like GAGs were also used to disrupt rosetting in line of previous evidence that subsets of DBL1α species bind to HS-like GAGs. Positive correlations between rosette disruption and invasion inhibition were achieved using the GAGs heparin and HS, both out competing the binding of PfEMP1 to heparane sulfate on the IE. The effect seen was higher in the hyper sensitive FCR3 parasites compared to the clinical isolates and in general higher than the effect of αIgM and αIgG rosette disruption. This implies that certain facets of invasion other than rosetting may be involved in and affected by GAG treatment. Bound IgG, IgM and α DBL1α antibodies were also visualized by iEM on the IE surface of a subset of the parasites.

All this taken together establishes a new paradigm in *P. falciparum* invasion studies, as well as the view on what factors contribute to the emergence of severe disease in the patient. Clearly PfEMP1 serves more than one role in malaria pathogenesis, not only in immune evasion and sequestration but also in facilitating invasion. Further studies of the different modalities in PfEMP1 facilitated invasion observed in this study will give valuable insight into what constitutes severe malaria.

6 CONCLUDING REMARKS

The data presented and discussed in this dissertation focuses on the special aspects of *P. falciparum* that may cause the severe malaria syndromes, what sets it apart from the parasite which causes the more common milder version of the disease. We have sampled *P. falciparum* of children, studied several different molecular aspects of the parasites and placed them into a molecular context with the aim of isolating features that characterize *P. falciparum* of severe malaria patients. The results illustrate how particular each parasite isolate is, in terms of expression of variable surface antigens at the infected erythrocyte surface, genomic features as well as adhesion, growth and multiplication capabilities. It was at first a somewhat daunting task to find a structure in the staggering variability of the system, yet we have found it is possible to identify certain molecular features of *P. falciparum* and correlate them to the different disease states of malaria. Still, many aspects of the parasite-host relationship have not been studied including the human side which may be just as complex as the part of the parasite.

Much of the exercise in categorizing the molecules presented on the erythrocyte surface by *P. falciparum*, including the efforts discussed here, is aimed towards finding suitable vaccine candidates for an erythrocytic stage vaccine. The quest for a vaccine effective in reducing malaria mortality has however been one lined with mainly frustration. Further, there is general consensus in the malaria community that a successful vaccine has to be composed of several components capable of raising multi-stage immunity.

On the other hand, promising results have been obtained in the form of a vaccine that targets the CSP protein present on the sporozoite surface. Subunit vaccines aimed at the merozoite surface have proven less successful. A reason for the latter could be that the merozoites in vivo are present in the blood stream very short periods of time before immunity is mounted to PfEMP1 because of rosetting assisted invasion. Another stumbling block has hitherto been the redundancy in the surface antigen repertoire. We herein provide a method to pick possible molecular targets out of the very large body of PfEMP1 sequences. However, the PfEMP1 region that we have investigated is comparably short relative to the full protein. Emerging methodologies to sequence full transcriptomes will in the future be of extreme importance to map entire variable surface antigen transcripts and their relative distribution in field isolates.

As we are presently in the post genomic era we have the tools within our grasp to unravel the great cipher of malaria variability. Still, one has to be reminded that the many faces of malaria appear different depending on a multitude of factors, for example geographic locations, human genotypes as well as immunological setup, malaria endemicity, nutritional status of the patient and access to heath care. In order to truly unthread the dependencies of what constitutes malaria these fundamental aspects have to be analyzed in union.

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