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Virulence in *Plasmodium falciparum* malaria:

mechanisms of PfEMP1-mediated rosetting

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ABSTRACT

Malaria is one of the most important infectious diseases in the world and the *Plasmodium falciparum* parasite is the causative agent of most of the severe cases. The pathogenesis of the disease is complex but sequestration and hence microvascular obstruction is associated with virulence of the parasite. Rosetting, the adhesion of a parasitized red cell (pRBC) to two or more non-parasitized RBC is central in the adhesion phenomena. The adhesin *Plasmodium falciparum* Erythrocyte Membrane Protein-1 (PfEMP1) mediates rosetting through its adhesive head structure composed of the NTS-Duffy Binding Like (DBL) 1α domain. Specific PfEMP1 antibodies (Abs) acquired after repeated exposures to parasites are associated with immunity to severe disease. In order to design effective therapies against severe malaria a deeper knowledge of the rosetting phenomenon is required.

A panel of monoclonal antibodies (mAbs) to NTS-DBL1α was generated by vaccination with recombinant protein. Epitopes recognized by the antibodies were mapped using a peptide array revealing that the reactivity of rosette disruptive monoclonal antibodies is localized in a specific region of subdomain 3 of DBL1α, independently of the parasite strain tested. In addition, the majority of anti-rosetting antibodies in a polyclonal IgG preparation towards NTS-DBL1α targeted the same area. This suggests subdomain 3 of NTS-DBL1α to be one of the major targets for rosette-disruptive antibodies. Further, generation of biologically active antibodies was consistent in different animal species and cross-recognition of heterologous rosetting domains was common in ELISA but not on live pRBC.

In parallel, to overcome the strain-specificity of the antibodies, a sequence motif present in subdomain 2 of the DBL1α sequence and previously associated with severe malaria was used for immunization. The peptide elicited a strain-transcending antibody response, with immune IgG recognizing a number of genetically distinct parasites, including both laboratory strains and patient isolates. Our results demonstrate the possibility to generate cross-reactive antibodies that recognize the pRBCs surface.

In addition, investigations were carried out on the naturally aquired human antibody repertoire as found in individuals living in an area of high malaria endemicity. Patients plasma samples were analysed for their biological activity towards a laboratory parasite strain. Findings were correlated with clinical symptoms and the epitopes recognized by the Abs on a peptide array. Reactivity of the plasma samples towards six of the peptides was correlated with the sample capacity to disrupt rosettes. The identified peptides were distributed along the NTS and DBL1α sequence, but mainly localized in subdomain 2.

Finally, by combining site directed mutagenesis with RBC binding and rosette inhibition studies, the localization of the binding site of one rosetting NTS-DBL1α domain was mapped to subdomain 2. Our results also demonstrate that rosetting inhibition by mAbs is not mediated by direct blockage of receptor binding but rather by modifications distal from the paratope.

In conclusion this thesis provides new insights into targets for vaccination-induced and naturally acquired antibodies towards PfEMP1-NTSDBL1α and it describes a receptor-binding site important for rosetting. Overall this thesis increases the knowledge on the molecular mechanisms underlying rosetting and could be helpful for the future rational development of therapeutic means against severe malaria.

LIST OF PUBLICATIONS

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

I. **Angeletti D,** Albrecht L, Blomqvist K, Quintana Mdel P, Akhter T, Bächle SM, Sawyer A, Sandalova T, Achour A, Wahlgren M, Moll K
Plasmodium falciparum rosetting epitopes converge in the SD3-loop of PfEMP1-DBL1α
PLoS ONE. 2012, 7(12):e50758

II. **Angeletti D*,** Albrecht L*, Wahlgren M, Moll K
Analysis of antibody induction upon immunization with distinct NTS-DBL1α-domains of PfEMP1 from rosetting *Plasmodium falciparum* parasites
Malaria Journal. 2013, Jan 24;12:32.

III. Blomqvist K, Albrecht L, Quintana Mdel P, **Angeletti D**, Joannin N, Chêne A, Moll K, Wahlgren M
A sequence in subdomain 2 of DBL1α of *Plasmodium falciparum* erythrocyte membrane protein 1 induces strain transcending antibodies
PLoS ONE. 2013, 8(1):e52679

IV. Albrecht L, **Angeletti D***, Moll K*, Blomqvist K, Valentini D, D´Alexandri F, Maurer M, Wahlgren M
Human anti-rosetting antibodies and B-cell epitopes of *Plasmodium falciparum* Erythrocyte Membrane Protein 1
Manuscript

V. **Angeletti D**, Sandalova T, Wahlgren M, Achour A
Receptor binding site of the *Plasmodium falciparum* rosetting domain NTS-DBL1α of PfEMP1
Manuscript

* Equal contribution

These publications were obtained during the course of education but are outside the scope of this thesis:

I. Nilsson S, Moll K, **Angeletti D,** Albrecht L, Kursula I, Jiang N, Sun X, Berzins K, Wahlgren M, Chen Q
Characterization of the Duffy-Binding-Like Domain of *Plasmodium falciparum* Blood-Stage Antigen 332.
Malaria Research and Treatment. 2011, 2011:671439

II. Nilsson S, **Angeletti D,** Wahlgren M, Chen Q, Moll K
Plasmodium falciparum antigen 332 is a resident peripheral membrane protein of Maurer's clefts.
PLoS ONE. 2012, 7(11):e46980.

III. **Angeletti D**, Kiwuwa MS, Byarugaba J, Kironde F, Wahlgren M
Elevated levels of high-mobility group box-1 (HMGB1) in patients with severe or uncomplicated *Plasmodium falciparum* malaria.
American Journal of Tropical Medicine and Hygiene. 2013 Apr;88(4):733-5.

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LIST OF ABBREVIATIONS

Ab	Antibody
ATS	Acidic terminal segment
CIDR	Cysteine rich interdomain region
CM	Cerebral malaria
CR1	Complement receptor 1
CSA	Chondroitin sulphate A
DBL	Duffy binding like
DBP	Duffy binding protein
EPCR	Endothelial protein C receptor
FACS	Flow activated cell sorting
FCS	Foetal calf serum
HS	Heparan sulphate
ICAM-1	Intercellular adhesion molecule-1
Ig	Immunoglobulin
mAb	Monoclonal antibody
MC	Maurer´s cleft
NTS	N-terminal segment
PAM	Pregnancy associated malaria
PECAM	Platelet endothelial cell adhesion molecule
PEXEL	*Plasmodium* export element
PfEMP1	*Plasmodium falciparum* erythrocyte membrane protein-1
pRBC	Parasitized red blood cell
PTEX	*Plasmodium* translocon of exported proteins
PVM	Parasitophorous vacuolar membrane
RBC	Red blood cell
SD	Subdomain
spp	Species
TNF	Tumour necrosis factor
TVN	Tubulovescicular network
ups	Upstream sequence
var	Gene encoding PfEMP1 protein
VCAM	Vascular cell adhesion protein
VSA	Variable surface antigen
VTS	Vacuolar transport signal
WHO	World Health Organization

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

1 INTRODUCTION

1.1 THE BURDEN OF MALARIA

Malaria is one of the most important infectious diseases that affects mankind worldwide. Risk of infection still exists in 99 countries. In 2010 the World Health Organization (WHO) reported 219 million new cases of malaria and 660,000 deaths, of these 80% and 91% were in sub-Saharan Africa, respectively (WHO, 2012). These numbers were recently questioned by Murray *et al.* in a report that suggested that WHO might be underestimating the number of deaths in patients over 5 years of age. The authors, by using verbal autopsies in their investigation, estimated the number of deaths at around 1,238,000, almost the double amount as compared to the WHO predictions (Murray et al., 2012).

Malaria represents an enormous socio-economical problem with high costs, both for individuals and governments (Sachs and Malaney, 2002), including costs for doctor-fees, antimalarial drugs, transportation, costs for the government to maintain health facility, provide insecticide-impregnated bed-nets, indoor-residual spraying and other preventive measures. Altogether these factors hamper economical development of countries that are already considered as "low-income", increasing their gap with developed countries (Sachs and Malaney, 2002). Tremendous efforts have been done in order to reach the goal of malaria control and elimination resulting in a 50% reduction of malaria cases between 2000 and 2010 (Alonso and Tanner, 2013). However the global economical crisis with the subsequent decrease in funding, in addition to the emergence of artemisinin resistance (Dondorp et al., 2009), poses a significant threat to the sustainability of the current situation. The discovery and use of long-lasting preventive measurements such as vaccines and/or other therapeutic interventions is therefore required.

1.2 PLASMODIUM SPECIES

Malaria is caused by unicellular eukaryotic protozoan parasites of the *Plasmodium* genus. Together with several other relevant human parasites (such as *Cryptosporidium* and *Toxoplasma*), these parasites belong to the Apicomplexa phylum, as they possess an apical complex containing unique organelles, such as micronemes and rhoptries, which allow them to invade host cells. There are over 250 *Plasmodium spp* that can infect a wide variety of vertebrate hosts including birds, reptiles, rodents and non-human primates. Among those only five can infect humans: *P. falciparum,* that causes the majority of severe cases, *P. vivax, P. ovale, P. malariae* and finally *P. knowlesi*, that has macaques as a natural host, but has been recently recognized as the fifth human malaria species (Cox-Singh et al., 2008; Jongwutiwes et al., 2004; Lau et al., 2011; Ng et al., 2008; Singh et al., 2004; White, 2008). The scope of this thesis is centred on *P. falciparum* therefore the focus of the following chapters will be mainly on this species.

1.2.1 Life cycle of Plasmodium spp.

The *Plasmodium falciparum* life cycle, similarly to most of the Apicomplexa, is complex and involves several hosts and parasites forms (Figure 1). *Plasmodia* are obligate intracellular organisms switching between mosquitoes (definite hosts) and humans (intermediate hosts). The female *Anopheles* mosquito transmits the parasite when taking a blood meal: while probing in search of a blood vessel it injects infectious sporozoites into the dermis. The injected sporozoites start moving by gliding motility within the dermis (Amino et al., 2006), this requires the sporozoites to traverse cell barriers, breaching the membrane of host cells (Amino et al., 2008; Mota et al., 2001), a process that continues for several hours (Yamauchi et al., 2007). Finally they penetrate blood vessels and are transported to the liver. Of the about 100 sporozoites injected by the mosquito only few will reach the liver while the majority is either destroyed in the skin or drained to lymph nodes where the adaptive immune response is initiated (Amino et al., 2006; Chakravarty et al., 2007; Yamauchi et al., 2007). Once in the liver the sporozoites traverse the sinusoidal barrier in order to invade the hepatocytes (Amino et al., 2008). At this stage, the parasite circumsporozoite protein (CSP) (Cerami et al., 1992; Frevert et al., 1993) plays a pivotal role and by sensing highly sulphated heparan-sulphate proteoglycans (HSPG) on hepatocytes, CSP will provide a signal to the parasite to stop migration and start invasion (Coppi et al., 2007). The micronemes proteins thrombospondin related adhesive protein (TRAP) (Ejigiri et al., 2012; Sultan et al., 1997) and apical membrane antigen (AMA1) (Silvie et al., 2004) play a key role in the invasive events. Invasion of the hepatocyte results in the formation of a vacuole inside the cell where the parasites can multiply (Mota et al., 2001; 2002). After 5-15 days, parasites-filled vesicles, called merosomes, are released by budding from hepatocytes into the sinusoids (Baer et al., 2007; Sturm et al., 2006).

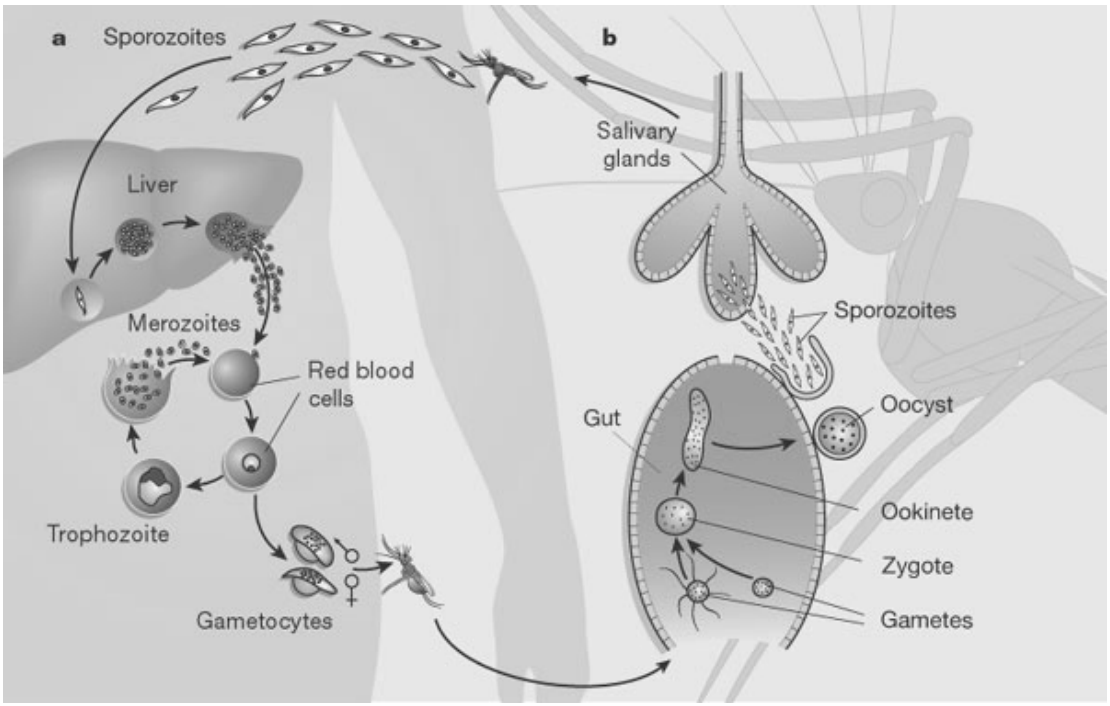

Figure 1. *Plasmodium falciparum* life cycle. (Adapted from Wirth, 2002 and reproduced with permission from Nature Publishing Group)

Following merosome rupture, thousands of merozoites are released into the bloodstream and are able to invade red blood cells (RBCs). RBC invasion is a complex process consisting of several steps that will be described and discussed in more detail in chapter *1.4.1*. In the newly invaded RBC the parasite starts an asexual developmental cycle (24-72 hours, depending on the *Plasmodium spp,* approximately 48 hours for *P. falciparum*): the parasites mature from ring through throphozoite to the final schizont stage. Schizont-parasitized RBCs release new merozoites into the circulation (6-24 depending on species, with 8-20 for *P. falciparum*) that will in turn invade new RBCs, leading to an exponential growth of the parasite in the bloodstream.
Not all parasites undergo asexual replication: a small subset, after schizogony, commit to sexual replication, differentiating into gametocytes (Bruce et al., 1990; Talman et al., 2004) with pre-determined sex (Silvestrini et al., 2000; Smith et al., 2000c). The molecular mechanisms of gametocytogenesis are not yet fully understood but the process is favoured by stimuli that have a detrimental effect on parasite growth, such as drug treatment (Buckling et al., 1999; Talman et al., 2004). Recent reports suggest that exosome-like vesicles and microvesicles are capable to provide signals in-between parasites that trigger the initiation of gametocytogenesis (Mantel et al., 2013; Regev-Rudzki et al., 2013). In *P. falciparum* it takes about 10 days for gametocytes to undergo differentiation into five distinct stages (Sinden, 1982): while early and late gametocyte stages are found in the bloodstream, intermediate-stages gametocytes are sequestered, possibly in the bone marrow (Day et al., 1998; Marchiafava and Bignami, 1892; Rogers et al., 2000; Smalley et al., 1981; Tibúrcio et al., 2013).
These sexual forms can be taken up by feeding mosquitoes thus completing the *Plasmodium* sexual cycle. The sudden change of microenvironment in the mosquito gut triggers a fast transformation of the male gametocyte into eight microgametes while the female simply exits from the RBC as rounded gamete. Only the fertilized zygote can survive and undergo differentiation in the hostile environment of the mosquito midgut, first to ookinete and then to oocyst (Sinden et al., 1985). Thousands of sporozoites break out from the oocyst and migrate into the mosquito salivary glands where they become fully infective and can be again injected into the human host (Touray et al., 1992; Vanderberg, 1975).

1.3 THE DISEASE

Out of the five species of *Plasmodium* that are infective to humans, *P. falciparum* is certainly the one that causes the overwhelming majority of severe cases and is responsible for most of morbidity and mortality. Although *P. ovale, P. malariae, P. vivax* and *P. knowlesi* are all thought to cause benign malaria, the latter two are being increasingly reported as causes of severe malaria (Anstey et al., 2009; Cox-Singh et al., 2008; Genton et al., 2008; Poespoprodjo et al., 2009; Singh et al., 2004).

Malaria is a heterogeneous disease with a great variety of symptoms and degree of severity. The infections are typically silent for a period of 8-12 days, which corresponds to the time of parasite hepatic development. Once the parasites are released into the blood circulation, the symptoms start typically as generalized flu-like manifestations and include, but are not limited to, fever, malaise, muscle ache, vomiting and diarrhoea. These first symptoms tend to correlate with increased number of parasites and are followed by periodical febrile attacks, known as malaria paroxysm

(with peaks every 24, 48 or 72 hours, depending on the length of the species life cycle). The malaria paroxysm has a sudden onset and usually starts with the patient feeling cold, despite having an elevated temperature, followed by rigor and sweating. For *P. falciparum* the paroxysm occurs every second day (tertian fever) but sometimes presents itself also as a less pronounced and continuous fever, due to the asynchronous nature of the parasites in circulation (Figure 2). The regular cycle and peaks of the fever generally correspond to the synchronous parasite bursting at the end of the erythrocytic cycle. Fever is caused by the release of pro-inflammatory cytokines, such as TNFα, and parasite-derived pyrogens concomitant to RBC destruction (Clark et al., 2006; Kwiatkowski et al., 1989; 1990). Other classical symptoms are splenomegaly, where the spleen enlarges as a response to acute infection, as well as hepatomegaly and haemolytic anaemia (Jakeman et al., 1999; Lamikanra et al., 2007).

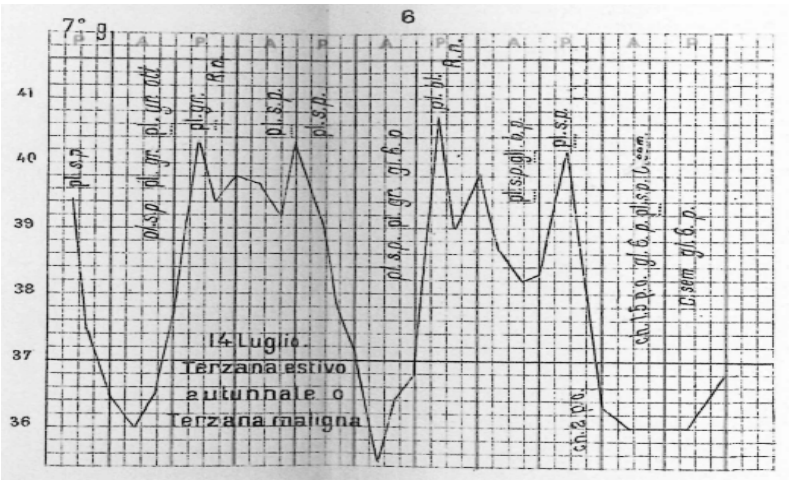

Figure 2. Malignant summer-autumnal tertian fever. Graph representing fever peaks of a patient, probably infected with *P. falciparum,* and showing the characteristic malaria paroxysm. (Adapted from Marchiafava and Bignami, 1892)

1.3.1 Severe malaria

In the past severe malaria was simplistically considered as either severe anaemia or cerebral malaria. Nowadays severe malaria is clearly recognized as a complex disease that may affect several organs, and the exacerbation of clinical symptoms by deregulated immune responses is well established (Mackintosh et al., 2004). Clinical features that, alone or in combination, lead to diagnosis of severe malaria are severe anaemia, unarousable coma (cerebral malaria), metabolic acidosis (respiratory distress), multiple convulsions, renal failure, circulatory collapse, hypoglycaemia, disseminated intravascular coagulation, placental infection, foetal loss and maternal anaemia. The course of the disease can be very fast and, if left untreated, almost always fatal. The mortality rate is high reaching from 15 to 20%, even if antimalarial treatment is administered.

1.3.1.1 Severe anaemia

Malaria-related anaemia is an important clinical manifestation of malaria. It is clinically defined as haemoglobin (Hb) concentration <50 g/l or haematocrit (Hct) <0.15 in the presence of *P. falciparum* parasitemia >10,000 parasites/µl. Anaemia is of

multifactorial origin and mainly due to an increased destruction of RBCs as well as a reduced RBC production (Menéndez et al., 2000). Although one could intuitively think that RBC destruction happens when parasites burst out from RBCs, this is only a marginal phenomenon compared to the enormous number of RBCs present in circulation. The presence of the parasite inside cells and parasite-factors released in circulation can activate phagocytosis of both parasitized and normal RBCs as well as trigger haemolysis (as a consequence of the deposition of complement-related factors and immunoglobulins on RBCs) (Lamikanra et al., 2007; Menéndez et al., 2000). Further, splenomegaly contributes to removal of RBCs and the expansion of the plasma volume (Angus et al., 1997). As mentioned previously, beside the active destruction of RBCs, the production of RBCs is significantly reduced. This phenomenon, known as dyserythropoiesis, is due to several factors ranging from direct erythropoietin (EPO) suppression due to cytokine imbalance, to disturbance of nuclear division in the bone marrow due to hypercellularity (Clark et al., 2006; Hassan et al., 1997).

1.3.1.2 Cerebral malaria

Cerebral malaria (CM) is one of the most severe complications of severe malaria and is clinically defined as unarousable coma in patients with *P. falciparum* parasitemia, after other causes of encephalopathy have been excluded (WHO, 2000). The fatality rate is between 15-20% and permanent neurological damages are common among those who survive (Birbeck et al., 2010). As many of the syndromes discussed in this chapter, the pathogenic mechanisms leading to CM are not yet fully understood.

Parasite sequestration, deregulation of immune responses and endothelial activation are probably the three key events leading to CM. However the exact order and their relative contribution in patients is hard to investigate and still matter of debate (Higgins et al., 2011). A recent study of fatal CM autopsies has provided a link between sequestration, vascular pathology and blood-brain barrier disruption, suggesting an intimate relation between intravascular and extravascular events leading to CM (Dorovini-Zis et al., 2011).

Sequestration of parasitized RBCs (pRBCs) in the microvasculature and subsequent reduction of the blood flow is certainly one of the most prominent features of cerebral malaria, and will be discussed in greater details in section *1.4.3*. Sequestration itself could be a cause of hypoxia or, more likely, critical reduction of both oxygen and nutrient supplies to the brain (Mishra and Newton, 2009).

Deregulation of the cytokine balance has also been observed in a number of studies, both using animal models as well as directly in patients suffering from CM. Elevated levels of pro-inflammatory TNFα, IFNγ, IL-6 and HMGB-1 (Alleva et al., 2005; Angeletti et al., 2013; Day et al., 1999; Kwiatkowski et al., 1990; Mishra and Newton, 2009) and/or decreased levels of anti-inflammatory IL-10 (Day et al., 1999) seem to exacerbate CM pathology.

Finally, immune responses also lead to endothelial activation and increase the number of adhesive molecules on endothelial cells, which in turn augments significantly pRBCs sequestration (Turner et al., 1994). In addition, activated endothelial cells release factors that may contribute to the loss of integrity of the blood-brain barrier (Higgins et al., 2011), a phenomenon that has also been demonstrated *in vitro* (Jambou et al., 2010).

1.3.1.3 Respiratory distress

For children older than two months of age respiratory distress is defined by a respiratory rate that reaches above 40 breaths per minute in conjunction with clinical signs of respiratory distress (WHO, 2000). It is tightly linked to metabolic acidosis and often results in fatal outcome (Marsh et al., 1995). Although the mechanisms are yet to be elucidated, it is well established that the pathology is exacerbated by a number of factors including secretion of parasite products and inflammatory cytokines as well as pulmonary parasite sequestration. Sequestration of parasites in the lungs might lead to reduced tissue perfusion, airway obstruction and pulmonary oedema factors that are all related to poor outcome (Haldar et al., 2007).

1.3.1.4 Pregnancy-associated malaria

The molecular details of pregnancy-associated malaria (PAM) will be described in section *1.4.3.1*. However, a general introduction of the pathology and its general mechanisms will be presented in this paragraph. PAM is a disease that primarily affects women during their first pregnancy, irrespective of previous malaria exposure. The outcome of the disease is generally low birth-weight and pre-term delivery, with a mortality estimate of 100,000 to 250,000 children per year (Duffy and Fried, 2011). Similarly to all other syndromes described in this chapter, PAM is determined by a combination of host and parasite factors which combined lead to the pathology (Umbers et al., 2011a).
The first observable phenomenon is extensive parasite sequestration in the intervillous space of the placenta (Fried and Duffy, 1996; Walter et al., 1982). pRBC accumulation in the placenta has several detrimental effects on the normal development of the organ. Firstly, it interferes directly with trophoblasts invasion of the uterus, a key step for appropriate placental function and vascularization (Rogerson and Boeuf, 2007). Secondly, sequestration can directly perturb hormonal production and nutrient transport (Boeuf et al., 2013; Umbers et al., 2011b). Finally, excessive sequestration causes deregulated activation of immune responses leading to placental inflammation, due to monocytes and fibrin deposition as well as cytokines and complement activation (Conroy et al., 2013; Ismail et al., 2000; Khattab et al., 2013; Suguitan et al., 2003).

1.3.2 Genetic factors

P. falciparum malaria has been defined as one of the greatest shaping factor of the human genome in recent times (Kwiatkowski, 2005). In fact, in sub-Saharan African populations several traits have been selected for, as they protect against the occurrence of severe malaria. The list includes heamoglobinopathies, glucose-6-phosphate dehydrogenase (G6PD) deficiencies and ABO blood group distribution (Kwiatkowski, 2005).
Normal haemoglobin A (HbA) is a tetramer with two α- and two β-globins chains. Three variants (HbCC, HbAS and α-thalassemia) protect against severe malaria syndromes (Taylor et al., 2012). HbC is due to a substitution of one single glutamate in the β-globin chain to a lysine while HbS has the same glutamate replaced by a valine. Although HbCC and HbAS carriers do not present major clinical symptoms, they have a selective advantage, in an endemic setting, as they are less susceptible to severe

malaria (Taylor et al., 2012). Similarly, α-thalassemia is a condition that reduces production of α-globin leading to decreased numbers of Hb tetramers with unpaired β-globin chains. This condition does not either result in major clinical symptoms, causing only mild anaemia, but protects from severe malaria. The mechanisms by which haemoglobinopathies protect against severe malaria are yet to be fully elucidated. However, several *in vitro* experiments have demonstrated impaired cytoadherence of Hb-variants-pRBCs (Cholera et al., 2008; Fairhurst et al., 2005), possibly due to abnormal knobs formation, deficient actin polymerization and consequent Maurer´s Cleft defects (see section *1.4.2*) (Cyrklaff et al., 2011; Kilian et al., 2013). A recent meta-analysis review has described how Hb modifications protect against severe malaria, but do not protect against uncomplicated malaria or parasitemia (Taylor et al., 2012). Altogether, these data suggest impaired cytoadherence and decreased sequestration to be the main protective factors in haemoglobinopathies (Fairhurst et al., 2012).

The ABO blood group system is the best characterized and best studied, as it is the most important system for blood group compatibility. Blood group A differs from O only by the addition of one extra N-acetyl galactosamine. Worldwide studies of blood group distribution indicated that while blood group A individuals are more prevalent in Nordic regions, the O blood group is prominent on the African continent. This suggests the presence of a selective force driving geographical blood group distributions (Cserti and Dzik, 2007). Epidemiological evidence, suggesting a protective effect of blood group O, have also been confirmed by two independent studies that elucidated the pathological and genetic mechanisms of increased malaria risk in non-O individuals (Fry et al., 2008b; Rowe et al., 2007). Here again, it seems that the augmented pathogenicity in blood group A individuals is due to high intravascular sequestration through the mechanism of increased rosetting (discussed in section *1.4.3.2*) (Barragan et al., 2000b; Rowe et al., 2007; Udomsangpetch et al., 1993).

1.4 MALARIA PATHOGENESIS

1.4.1 Erythrocyte invasion

After completing its 48 hour life cycle the parasite needs to invade new RBCs in order to propagate. Erythrocyte invasion by merozoites is a quick multistep process that takes about 20 seconds to complete. The five steps of invasion are: attachment, reorientation, junction formation, invasion and post-invasion (Cowman et al., 2012). Before egress both the parasitophorous vacuolar membrane (PVM) and the RBC membrane need to be disrupted. A number of proteases are involved in this process that are necessary for correct merozoite egress (Roiko and Carruthers, 2009). During attachment any part of the merozoite binds to one RBC in a low-affinity interaction (Dvorak et al., 1975); the proteins involved are unknown but this step involves major movement of merozoites and rearrangement of erythrocyte membrane (Gilson and Crabb, 2009).
After the initial attachment the merozoite reorient and interacts with the RBC by its apical end. This irreversible attachment involves proteins of the erythrocyte binding like (EBL) and reticulocyte homology ligand (Rh) families to a number of diverse host cell receptors (Tham et al., 2012). All the members of these protein families are dispensable, suggesting a redundancy of attachment pathway. However the Rh5 protein

that interacts with the red cell protein BASIGIN, has been recently shown to be strictly necessary for parasite invasion (Crosnier et al., 2011).
Junction formation involves the movement of apical membrane 1 antigen (AMA1) to the merozoite surface through its interaction with rhoptry neck protein 2 (RON2). The RON proteins are secreted into the RBC membrane and accumulate into the cytoplasm with just a small extracellular portion that is important for interactions with AMA1 (Riglar et al., 2011; Srinivasan et al., 2011; Tonkin et al., 2011). This newly formed link between parasite and RBC likely triggers the release of the rhoptry bulb providing proteins and lipids required for parasitophorous vacuole formation and facilitating the next invasion step (Riglar et al., 2011). During the invasion itself, some unknown factors stimulate the actin-myosin motor so that the junction moves from the anterior to posterior end of the merozoite, bringing the merozoite into the RBC (Aikawa et al., 1978; Angrisano et al., 2012). Finally the vacuole reseals behind the merozoite, in the last and crucial step for parasite survival, driven by hitherto unknown mechanisms (Cowman et al., 2012).

1.4.2 Protein transport and host cell remodelling

P. falciparum invades human RBCs, terminally differentiated cells that are devoid of most of the normal organelles and functions common to other cells. Therefore, the parasite must be able to remodel and modify these cells in a way that is optimal and beneficial for its survival in the human body. After invasion of RBCs, the parasite remains enclosed by the PVM at any time of its intracellular growth (Aikawa et al., 1978). The parasite will then build a complex trafficking network that will distribute proteins to their subcellular location emanating from the PVM, namely the tubolovescicular network (TVN) (Atkinson and Aikawa, 1990). All proteins will consequently need to cross three distinct membranes in order to be exported into the host cell cytoplasm, the first being the parasite membrane (Boddey and Cowman, 2013). Most of the exported proteins comprise a hydrophobic signal, localized 20-60 amino acids from the N-terminus, that allows them to enter the endoplasmic reticulum and thus initiate the secretory pathway (Waller et al., 2000). After fusion of the cargo vesicles with the plasma membrane, the proteins are localized in the parasitophorous vacuole (PV) and thus need to cross the PVM in order to reach the RBC cytosol. A pentameric sequence, localized 20-30 amino acids after the first signal sequence and named *Plasmodium* export element (PEXEL) (Marti et al., 2004) or vacuolar targeting sequence (VTS) (Hiller et al., 2004) is required for export of most parasite proteins. The PEXEL/VTS motif is recognized and the N-terminus of the molecule cleaved off within the endoplasmic reticulum by a resident protease, plasmepsin V (Boddey et al., 2010; Russo et al., 2010), directing thereafter the mature protein to the host cell (Boddey et al., 2010; Russo et al., 2010). All the proteins that possess a PEXEL/VTS motif form the so-called *P. falciparum* exportome, the largest among all studied *Plasmodia* species (Sargeant et al., 2006; van Ooij et al., 2008). However, a fraction of exported proteins, including *Plasmodium falciparum* Membrane Protein-1 (PfEMP1), carry neither a hydrophobic signal sequence nor a PEXEL motif and can thus not be cleaved by plasmepsin V (Boddey et al., 2013). Whether these proteins share common exporting mechanisms remains unclear and is currently subject of investigation. Once in the PV, proteins need to cross the PVM and they do so via a protein transport translocon. The *Plasmodium* translocon of exported proteins (PTEX) is a multimeric

complex composed of at least five members, which facilitates the passage of proteins to RBC cytoplasm (de Koning-Ward et al., 2009; Riglar et al., 2013). There is compelling evidence that most proteins, once in the PV, are unfolded, transported through the PTEX in a soluble state and thereafter associated with chaperone proteins in the host cell cytoplasm (Gehde et al., 2009; Grüring et al., 2012; Külzer et al., 2012; Papakrivos et al., 2005).
Another important structure that will appear early after invasion, at the beginning of ring stage development (Grüring et al., 2011), are Maurer´s Clefts (MC). Proteins associated with virulence of the parasite concentrate in these disc shaped organelles, that arise from the PVM and are connected to the TVN (Aikawa et al., 1986; Haeggström et al., 2007; Wickert et al., 2004). They are thereafter transported altogether to the RBC membrane (Bhattacharjee et al., 2008). Although it is still unclear how these proteins are transported to and from MC, there is increasing evidence that also insoluble proteins are transported into MC in soluble state (Grüring et al., 2011; 2012; Külzer et al., 2012; Papakrivos et al., 2005). Loaded MC subsequently move along the TVN and migrate to the RBC periphery where they tether to the membrane approximately 16-20 hours post invasion (Grüring et al., 2011). Several proteins are of importance for the formation of MC including *P. falciparum* skeleton binding protein (PfSBP1) that links the MC to cytoskeleton, the Membrane-associated His-rich protein (MAHRP) that is possibly involved in PfEMP1 loading into MC, the Ring exported protein-1 (REX1) that sculptures the organelles, PfEMP3 that binds to both spectrin and MC and contributes to increased rigidity of pRBCs and also Pf332 that binds cytoskeleton and increases pRBCs rigidity (reviewed in (Maier et al., 2009))
Finally, knob-like protrusions start forming on erythrocytes surface at approximately 16 hours after invasion (Gruenberg et al., 1983). The essential protein for this process is the Knob-associated histidine rich protein (KAHRP) (Crabb et al., 1997). KHARP is probably an important virulence factor *in vivo* since its presence is associated with stronger cytoadhesion under flow (Crabb et al., 1997), however it is not necessary for correct PfEMP1 presentation on the pRBC surface (Biggs et al., 1989; Udomsangpetch et al., 1989a).

1.4.3 Sequestration

"*The adult and sporulated forms tend to accumulate in the capillaries, especially in the brain, where, in the minute lumen, huge are the circulatory hindrances…"*
"…the red blood cells invaded by the parasite generate more resistance to the circulation as compared to the normal ones…they (RBCs) slow down or stop in some capillaries, in which the degenerative alterations of the endothelium, derived from the circulatory defects, become reason of new stagnation"
(Marchiafava and Bignami, 1892)

Already in 1892 severe malaria was well studied in the Roman countryside. Italian scientists Marchiafava and Bignami meticulously followed and documented a number of malaria cases, observing the pathological features of the disease and elucidating the pathophysiological processes leading to the severe disease (Marchiafava and Bignami, 1892). After more than one hundred years it is still recognized that one of the key pathological feature of severe malaria is parasite sequestration in the microvasculature (Dondorp et al., 2008; Hanson et al., 2012; Ponsford et al., 2012; Seydel et al., 2006;

Taylor et al., 2004; White et al., 2013), as extensively discussed in the previous section. In this paragraph the focus will mainly be on molecular interactions and mechanisms leading to sequestration.
In blood samples taken from patients mature forms of parasites are rarely found as they sequester in the deep microvasculature of different organs (Marchiafava and Bignami, 1892). This measure is taken by the parasite in order to avoid splenic clearance and is made possible by the drastic modifications of the RBCs and consequent surface exposure of parasite´s surface antigens. Sequestration can be mediated by direct binding of pRBCs to endothelial cells of the capillaries (cytoadhesion) or by binding of pRBCs to two or more non-parasitized RBCs (rosetting) (Rowe et al., 2009). Evidence from splenectomised patients demonstrate that in the absence of the spleen sequestration in the microvasculature is absent (Bachmann et al., 2009).

One of the major hurdles in studying sequestration is the lack of suitable animal models (beside aotus and squirrel monkeys) that mimics the phenomenon as seen in humans. Most of the studies to confirm the molecular basis of sequestration have adopted either one of the following two approaches. The first is to study parasites freshly isolated from patients for their adhesive characteristics and associate those with clinical symptoms or disease severity. Adhesion of pRBCs is tested in static or flow assay on endothelial cells or purified receptors coated on plastic/glass/beads. The second is to investigate human population, living in endemic areas, for genetic polymorphisms. Mutations, which downregulate or modify the adhesive receptors, are investigated for their linkage with protection from parasitemia, clinical symptoms or severe disease.

1.4.3.1 Cytoadhesion

Cytoadhesion is the binding of pRBCs to endothelial cells and is predominantly mediated by the parasite variant surface antigen PfEMP1 (Baruch et al., 1995). This phenomenon has been shown to be associated with severe disease both directly in patients (Dondorp et al., 2008) as well as in post-mortem studies (Dorovini-Zis et al., 2011; Ponsford et al., 2012). Recently, a subset of PfEMP1 has been identified, by three independent research groups, as responsible for adhesion to brain endothelial cells and associated with severe malaria (Avril et al., 2012; Claessens et al., 2012; Lavstsen et al., 2012). Several host receptors have been studied for their involvement in pRBCs cytoadhesion and the best characterized will be discussed below. It has to be noted, however, that such a complex process probably involves several receptors binding synergistically, in a manner that mimics the three-step leukocyte recruitment (Ho et al., 2000; McCormick et al., 1997; Yipp et al., 2000).

CD36
The scavenger receptor CD36 is expressed on a variety of human cells including macrophages, monocytes, platelets and endothelial and epithelial cells (Greenwalt et al., 1992). It was one of the first human receptor shown to interact with parasite proteins and its adhesion with PfEMP1 has been extensively studied: the interaction site both on CD36, as well as the specific PfEMP1-domain (CIDRα) have been mapped. Further, only PfEMP1 of group B and C are interacting with CD36 (Baruch et al., 1999; Klein et al., 2008; Miller et al., 2002; Robinson et al., 2003).

Most of clinical isolates are able to bind to CD36 (Oquendo et al., 1989). However, association of CD36 binding with disease severity seems to be dependent on the geographical area with no association found in isolates from Africa and conflicting results with isolates from Asian patients (Rowe et al., 2009). Studies on CD36 human polymorphisms report a positive selection for a CD36 nonsense variant in African population, however after some contradictory reports it seems clear that there is no association between such a variant and protection from severe malaria (Fry et al., 2009). The only study performed in Asia suggests linkage between polymorphism and protection, consistently with results from binding assays (Omi et al., 2003).

ICAM-1

CD54 or intercellular adhesion molecule-1 (ICAM1) is expressed on endothelial cells and leukocytes (Berendt et al., 1989). pRBCs interaction with ICAM1 is PfEMP1-mediated (Smith et al., 2000a). For this interaction the fine molecular details have been elucidated: PfEMP1 variants encoded by group B *var* genes are responsible for adhesion via their DBLβ-C2 domain (Chattopadhyay et al., 2004; Howell et al., 2008; Janes et al., 2011).

As for CD36, association of ICAM1-binding with disease state resulted in contradictory data where a number of studies that measured pRBC binding to ICAM1 in static condition failed to identify any association with disease state (Heddini et al., 2001a; Newbold et al., 1997; Rogerson et al., 1999). However, a more recent study, that compared isolates derived from uncomplicated malaria versus cerebral malaria patients employing a flow-based binding assay, demonstrated higher ICAM1-binding capacity in the isolates from the latter group (Ochola et al., 2011). Further, autopsies show co-localization between sequestered pRBCs in microvasculature with ICAM1 (Turner et al., 1994). Similarly to the binding data, the search for human polymorphisms in the ICAM1 gene has not provided any proof for selective pressure neither in the African nor in the Asian population (Cserti-Gazdewich et al., 2012; Fernandez-Reyes et al., 1997; Fry et al., 2008a). These conflicting results might just be a reflection of the complex binding events that occur *in vivo*, that could also possibly involve multiple receptors both on the endothelium as well as on the erythrocyte.

EPCR

Endothelial protein C receptor (EPCR) is expressed on endothelial cells in most tissues. EPCR binds protein C and enhances its activation by the membrane thrombin-thrombomodulin complex (Stearns-Kurosawa et al., 1996). It has recently been described that a subset of PfEMP1, expressing Domain Cassettes (DC) 8 and 13 and associated with severe malaria, mediates the binding of pRBCs to endothelial cells derived from brain microvasculature (Avril et al., 2012; Claessens et al., 2012; Lavstsen et al., 2012). Screening of over 2,500 human plasma membrane proteins against a specific DC8-PfEMP1 identified EPCR as responsible for this interaction with CIDRα1 domain being the minimal binding region (Turner et al., 2013). Similarly, CIDRα1 was confirmed as the only PfEMP1-domain able to inhibit pRBCs binding to brain endothelial cells (Avril et al., 2013).

Almost simultaneously, another study investigated *post mortem* staining of brain sections from CM patients and identified a loss of EPCR in microvessels where parasite sequestration was more pronounced. In addition EPCR loss was co-localized with disturbance of coagulation and inflammation (Moxon et al., 2013). Altogether these

data suggest a mechanism by which sequestered parasites decrease the level of host-cell surface EPCR and simultaneously bind to the remaining receptor having the overall effect of decreasing activated protein C (APC), a cytoprotective protein (van der Poll, 2013). Interestingly, several EPCR polymorphisms are documented and one of them is known to increase the amount of circulating soluble-EPCR (sEPCR) (Medina et al., 2004; Saposnik et al., 2004). Since sEPCR is able to dislodge PfEMP1 binding to brain endothelial cells (Turner et al., 2013), it could be interesting to investigate the presence of the mentioned polymorphism in African population and its association with CM protection. Further, it could be worth to investigate the effect of APC in severe-malaria treatment.

PECAM1

PECAM1 is expressed on a variety of cells, including endothelial cells. pRBCs from patient isolates as well as laboratory strains adhere to PECAM1 via PfEMP1 (Chen et al., 2000; Heddini et al., 2001a; Treutiger et al., 1997). To date, no correlation has been shown between adhesion and disease severity (Heddini et al., 2001b). Recently DC5-containing PfEMP1 have been identified as the subset responsible for this adhesion, and antibodies against DC5-PfEMP1 correlate with protection from febrile malaria and higher haemoglobin levels (Berger et al., 2013).

Heparan sulphate

Heparan sulphate (HS) is found on most cell types, including endothelial cells in the microvasculature (Vogt et al., 2003). The parasite mediates its interaction with HS via the N-terminal portion of PfEMP1, and more precisely the DBL1α domain (Chen et al., 1998a; Vogt et al., 2003). Heparin, a highly sulphated version of HS, requires N-, 6-O-, and 2-O- sulphation, and should be at least 12 mers in order to bind to PfEMP1 (Barragan et al., 2000a). Patient isolates are able to bind HS on endothelial cells (Vogt et al., 2003). Association with severe disease has been shown in one study with African isolates where fluorescently-labelled heparin bound predominantly to isolates from severe malaria patients (Heddini et al., 2001b). Studies linking HS polymorphisms to disease severity are missing but, for the first time, a genetic variation study in genes involved in HS biosynthesis revealed that some mutations are associated with increased parasitemia (Atkinson et al., 2012). However it has to be noted that in this study the clinical signs of the disease are not taken into account. Future studies are needed to link the same polymorphisms to different disease state.

CSA

Chondroitin sulphate A (CSA) is a sugar that is not expressed on human cells under normal conditions. However, the syncytiotrophoblasts of the placenta, which develops during pregnancy, are rich in CSA and parasites take advantage of the newly expressed receptor in order to sequester and escape immune clearance (Fried and Duffy, 1996). The parasite receptor is a specific PfEMP1 variant, called VAR2CSA (Salanti et al., 2004). Extensive studies have been performed on the molecular interactions between these two molecules: full length VAR2CSA is required for high affinity CSA binding and the protein assumes an overall spherical conformation (Khunrae et al., 2010; Srivastava et al., 2010) with the minimal core binding domain mapped to the N-terminal DBL2x (Clausen et al., 2012; Srivastava et al., 2011). In addition, pRBCs from pregnant women bind non-immune immunoglobulin (Ig), possibly bridging

pRBCs to syncytiotrophoblasts (Creasey et al., 2003; Flick et al., 2001; Rasti et al., 2006).

Other receptors (P-selectin, Thrombospoindin)
A number of other receptors have been suggested to be involved in parasite cytoadhesion. For most of them the number of studies remains scarce and the link with severe disease has not been established. Two of these recptors will be briefly discussed below.
Thrombospondin (TSP) is an adhesive protein released into the plasma by platelets. It was the first adhesive receptor identified for pRBCs (Roberts et al., 1985), with a number of isolates being able to bind it when expressed on endothelial cells (Heddini et al., 2001b; Roberts et al., 1985). The parasite binding is controversial. There seems to be no association between TSP binding and disease severity.
P-selectin is expressed on activated platelets and endothelial cells. It seems to have a role in pRBCs rolling and in facilitating adhesion to CD36 (Udomsangpetch et al., 1997). The parasite binding partner is PfEMP1 (Senczuk et al., 2001). No studies have been performed correlating binding with severe disease.

1.4.3.2 Rosetting

Rosetting is defined as the binding of one parasitized RBC to two or more non-parasitized RBCs (Figure 3). This phenomenon has been observed since the 1980s with varying degrees between clinical isolates and laboratory strains (Carlson et al., 1990; David et al., 1988; Treutiger et al., 1992; Udomsangpetch et al., 1989b; Wahlgren et al., 1992). In experimental models, rosetting enhances microvascular obstruction (Kaul et al., 1991) and this parasite phenotype has been associated with disease severity in studies in Africa (Carlson et al., 1990; 1994; Doumbo et al., 2009; Rowe et al., 1995).

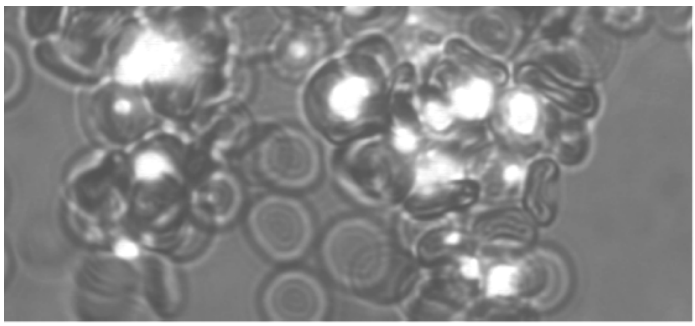

Figure 3. Rosetting in FCR3S1.2 pRBCs, with trophozoites (in white) binding to several RBCs. Image courtesy of Kirsten Moll.

Rosetting is mediated by the parasite ligand PfEMP1 and, in particular, by the semi-conserved head structure that includes the N-Terminal Sequence (NTS) and DBL1α. PfEMP1 that have the ability to mediate rosetting are mostly encoded by group A *var* genes (discussed in *1.5.4.1*) (Albrecht et al., 2011; Rowe et al., 1997; Vigan-Womas et al., 2008). So far complement receptor-1 (CR1), blood group A (BgA) and HS have been identified as rosetting receptors. Serum proteins also play a fundamental role in rosette formation. Other than sequestration, one possible role for rosetting could be the enhancement of invasion by bringing cells close to each other thereby facilitating merozoite invasion. Although there is still no evidence *in vitro* for this hypothesis

(Clough et al., 1998a), other studies on clinical isolates and *in vivo* using monkeys have demonstrated a link between rosetting rate and increased parasitemia possibly supporting this notion (Le Scanf et al., 2008; Rowe et al., 2002a).

Complement receptor 1

Complement receptor 1 (CR1) is a complement protein found on RBCs, leukocytes and dendritic cells. A number of laboratory and clinical isolates are unable to form rosettes with CR1-deficient RBCs, demonstrating the importance of this receptor in these adhesive events (Rowe et al., 1997). Further, anti-CR1 mAb are able to revert already formed rosettes (Rowe et al., 2000). Interactions occur via the central and C-terminal regions of the DBL1α domain of PfEMP1 (Mayor et al., 2005). Cockburn *et al* report that CR1-deficient RBCs has been selected for in a high-transmission area in Papua New Guinea and that CR1-deficiency correlates with severe disease protection (Cockburn et al., 2004). However, more recent data suggest that malaria is not the driving force in Knops blood group polymorphism (resulting from mutations in CR1 gene) (Tetteh-Quarcoo et al., 2012).

Blood group A

Blood group A (BgA) is a saccharide present on the RBC surface (in BgA individuals). It differs from H (found on BgO individuals) by the addition of an extra N-acetyl galactosamine at the end of the sugar chain. Rosetting parasites have a preference for BgA or BgB RBCs, forming smaller and looser rosettes with BgO RBCs (Barragan et al., 2000b; Carlson and Wahlgren, 1992; Carlson et al., 1994; Chotivanich et al., 1998; Rowe et al., 1995; 2007; Treutiger et al., 1999; Udomsangpetch et al., 1993). BgA trisaccharides can disrupt rosettes formed by parasites with RBCs of the corresponding blood group (Barragan et al., 2000b; Carlson and Wahlgren, 1992). The interacting partner is the NTS-DBL1α domain of PfEMP1, and for one PfEMP1-variant the molecular mechanism has been elucidated (Vigan-Womas et al., 2012). BgO has been shown to be a protective trait in African population, with driving forces towards variants in the ABO glycosyltransferase gene (Fry et al., 2008b). Further, a study performed in Mali proved that BgO mediates protection to severe malaria via the mechanism of reduced rosetting (Rowe et al., 2007).

Heparan sulphate

HS is present on RBCs (Vogt et al., 2004) and earlier experiments showed that treatment of RBCs with glycosaminoglycan-removing enzymes disrupted rosettes *in vitro* (Chen et al., 1998a). Heparin, a highly sulphated version of HS, has been shown to interact directly with PfEMP1-NTS-DBL1α of two different parasite variants (Barragan et al., 2000a; Juillerat et al., 2010). However for the PAvarO variant, HS is not the main receptor on RBCs (Juillerat et al., 2010). Heparin and heparin derivatives can disrupt rosettes both in laboratory strains and in patient isolates (Carlson and Wahlgren, 1992; Leitgeb et al., 2011; Rowe et al., 1994; Udomsangpetch et al., 1989b).

Serum proteins

Serum proteins and in particular non-immune IgM are of fundamental importance for the formation of rosettes. While the role of IgG is unclear (Flick et al., 2001; Rowe et al., 2002b; Treutiger et al., 1999), IgM binding has consistently been reported to be a key factor for rosette formation both in laboratory strains and in patient isolates

(Clough et al., 1998b; Czajkowsky et al., 2010; Ghumra et al., 2008; Rowe et al., 2002b; Scholander et al., 1996; Treutiger et al., 1999). The role of IgM is not limited to the enhancement of rosette formation but these immunoglobulin are also involved in immune evasion by masking IgG epitopes from neutralizing antibodies (Barfod et al., 2011). The terminal domains of PfEMP1 DBLζ and DBLε are mediating the interaction of pRBCs with the Cμ4 domain of IgM (Ghumra et al., 2008; Semblat et al., 2006). Beside immunoglobulins, other serum proteins, such as von Willebrand factor, fibrinogen, complement factor D and albumin all play an important role in rosette formation, but their binding partners are currently unknown (Luginbühl et al., 2007; Treutiger et al., 1999).

1.5 VARIANT SURFACE ANTIGENS

1.5.1 Antigenic variation

Antigenic variation is a mechanism of survival adopted by many infectious organisms. In a complex landscape, such as the human body and its multiple defence mechanisms, pathogens must be able to interact with the host via surface expressed ligands without being recognized by the immune system. By utilizing antigenic variation, pathogens can alter their antigenic coat, therefore increasing fitness advantage and augmenting their evolutionary success. Most of the antigens that undergo variation are surface proteins that are involved in host-pathogen interaction (Deitsch et al., 2009).
Indeed, the first and more intuitive benefit is the possibility to evade immune response: without the ability to change surface molecules, the pathogen would be rapidly neutralized by antibodies and/or phagocytized. The capacity to turn off or switch antigen-variant on the surface renders the immune system ineffective and to protect against pathogenic organisms, such as *Borrelia* and *Plasmodium* (Badell et al., 2000; Cadavid et al., 1994).
The second advantage of antigenic variation is the ability to persist inside the host. Vector-transmitted pathogens (such as *Plasmodium, Babesia, Borrelia* and *Trypanosoma*) need to persist for weeks in the bloodstream in order to maximize their chances to be uptaken by a new vector.
Finally antigenic variation enables the micro-organisms to re-infect hosts that have been previously cured, giving the pathogen a larger susceptible population and increasing its evolutionary chances. Further, multiple infections by antigenically variant organisms of the same species can favour the exchange of genetic material between them (Futse et al., 2008). Overall, antigenic variation provides the pathogens with the ability to escape, persist and re-invade.
There are two main mechanisms by which an antigen undergoes variation. Either by "random" or unprogrammed variation (point mutations and simple gene recombination) or by programmed variation or antigenic variation *sensu stricto* that involves complex switching and silencing mechanisms between multiple genes of the same family (Deitsch et al., 2009).
Random variation is a direct consequence of imprecise DNA replication, repair and recombination and it is a mechanism preferentially adopted by RNA viruses (Drake and Holland, 1999). Many viruses, such as hepatitis and influenza, have an enormous mutation rate contributing to their capacity to establish either chronic infections or favour re-infection. An interesting example is the case of influenza, an RNA virus with

great mutation rate, which does not need antigenic variation to persist in the host but adopts this strategy to be able to infect population wide and re-infect in different seasons (Blackburne et al., 2008).
Antigenic variation *sensu strictu* involves more complex mechanisms and can be further sub-divided into genetic (such as recombination) and *in situ* (transcriptional, translational and epigenetic) mechanisms.
The most common mechanism involving DNA recombination is termed gene conversion and consists of the movement of non-expressed coding regions into defined expression sites. Either the whole gene or small parts, creating chimeric sequences, can be moved. This system is used by the bacteria *Borrelia* (*vlp, vsp* and *vlsE)* (Kitten and Barbour, 1990; Zhang and Norris, 1998) and *Neisseria* (*pil*) spp. (Haas and Meyer, 1986), as well as the eukaryotic African trypanosome (*vsg*) (Bernards et al., 1981).
In *Borrelia* the vlp- and vsp- proteins are surface lipoproteins with unknown function but are a major target for the immune system. However they also undergo antigenic variation and consequently become difficult targets for neutralizing antibodies (Norris, 2006). There is one active locus with one transcribed gene and recombination happens in a hierarchical manner with silent *vlp* and *vsp* loci. The tight regulation depends on the homology of the upstream and downstream regions, allowing a semi-programmed antigenic variation, associated with fever relapses in Borreliosis (Dai et al., 2006).
Millions of variant surface glycoproteins (VSG) coat the surface of *T. brucei* making it impossible for antibodies to target any other protein. Specific response to one VSG is mounted rapidly but at each division the parasite can switch and change surface VSG (Schwede and Carrington, 2010). The most common expression mechanism occurs through an active subtelomeric expression site: of the 1000 *vsg* per genome, present either on minichromosomes or in the central chromosome parts, one is expressed at the time. Via gene conversion one *vsg* is repositioned to the subtelomeric expression site at the time, giving almost unlimited surface variability to the pathogen (Taylor and Rudenko, 2006).
Transcriptional and translational control are adopted by many bacteria and protozoa. In *Neisseria, E.coli* and *Candida,* transcription can be regulated by the length of repeats in the promoter or alternatively by the number of repeats in the coding sequence (van der Ende et al., 1995). The repeats determine whether the full-length protein will be translated or the product will result into a truncated protein. In *Giardia lamblia* it has been demonstrated that antigenic variation can be determined by post-transcriptional control. Although many variant surface protein (*vsp*) genes are transcribed, only one is stable and expressed at the time, while others are silenced through RNA interference mechanisms (Prucca et al., 2008). This ingenious regulation enables the parasite to switch rapidly between expressed VSP during the course of infection.
Finally the last and probably most complex and fascinating antigenic variation mechanism is through epigenetic changes. Changes in chromatin structure and nuclear organization are adopted, especially by eukaryotic pathogens, in order to regulate mutually exclusive expression. The regulation in African trypanosome represented for a long time a paradigm for epigenetic regulation, although this mechanism was later found to play only a minor role. In *Trypanosoma* the subtelomeric expression sites can be changed from silent to transcriptionally active via epigenetic changes, with silent expression sites having more condensed chromatin and higher number of methylation (Taylor and Rudenko, 2006). *var* genes of *P. falciparum* are mutually exclusive and regulated mainly at the level of transcription initiation)details will be discussed in

section *1.5.4*). Briefly, nuclear positioning, methylation and acetylation regulate transcription of *var* genes. *Giardia* and *Babesia* have similar epigenetic modifications and regulations (Kulakova et al., 2006).
Candida albicans also adopts an epigenetic mechanism with subtelomeric regions causing changes in chromatin organization and, in concert with acetylation and de-acetylation, providing a basis for genic switching (Domergue et al., 2005).
Overall, antigenic variation is an array of complex phenomena utilized by pathogens, with most of them not exclusively adopting one strategy but a combination of the above, to counteract the defence strategies employed by the immune system.

1.5.2 PfEMP1

The variable surface polypeptide *Plasmodium falciparum* erythrocyte membrane protein 1 (PfEMP1) was initially identified in the mid-eighties by surface iodination experiments (Leech et al., 1984). It was shown that presence of variant surface antigens (VSA) on infected erythrocyte surface would confer adhesive properties to different cell types (David et al., 1983). Ten years later, the family of genes encoding PfEMP1 was discovered and termed *var* (Baruch et al., 1995; Smith et al., 1995; Su et al., 1995).
PfEMP1 is a multidomain protein that varies in size between 250 and 350 kDa. It consists of multiple tandemly arranged Duffy Binding Like (DBL) domains and Cystein-Rich Interdomain region (CIDR), with an N-terminal sequence (NTS) present in the majority of PfEMP1 (Figure 4A). About 95% of PfEMP1 proteins have a semi-conserved head structure consisting of the NTS, a DBLα and CIDR domain (Rask et al., 2010). Following a transmembrane region the acidic terminal sequence (ATS) is present inside the RBC (Su et al., 1995).

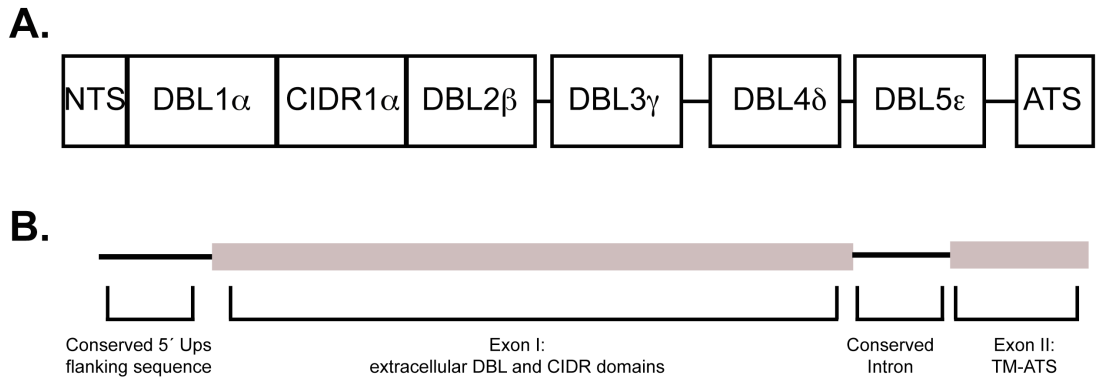

Figure 4. Schematic representation of PfEMP1. Multi-domain protein structure (A) and gene structure of *var* gene (B)

Conventionally the DBL and CIDR domains are numbered consecutively starting from the N-terminal domain and have been classified into six different DBL types (α, β, γ, δ, ε and ζ) and five CIDR types (α, β, γ, δ and pam) according to common sequence similarities (Rask et al., 2010). The analysis of PfEMP1 diversity in seven genomes by Rask and colleagues has enabled the identification of common features in distinct PfEMP1 proteins and in particular of domain cassettes (DC) defined as " two or more consecutive domains belonging to particular subclasses and present in three or more genomes" (Rask et al., 2010). The DC division offers a good basis for interpretation and design of experimental studies and has already been used for the identification of a

number of receptor-binding signatures (Bengtsson et al., 2013; Berger et al., 2013; Lavstsen et al., 2012; Turner et al., 2013). Further Rask *et al* re-defined homology blocks (HB), first described by Smith *et al.* (Smith et al., 2000b), as conserved sequence signatures present in all the DBL and CIDR domains. 628 HB were identified and numbered according to frequency of occurrence (Rask et al., 2010). Five HB, that were the same as described by Smith, are conserved in the majority of DBL domains while three are conserved in the CIDR domains (Rask et al., 2010; Smith et al., 2000b).

1.5.3 *var* genes genomic organization

Each parasite genome comprises approximately 60 *var* genes, localized mainly in the highly polymorphic chromosome end regions with few of them in the central chromosome portion (Gardner et al., 2002; Hernandez-Rivas et al., 1997). The majority of *var* genes are localized in subtelomeric regions adjacent to the non-coding telomere repeat elements (TARE 1-6). Frequently one to three *var* genes exist either in a tail-to-tail orientation with one or more *rif* genes in between or clustered together, followed by a number of *rif* and *stevor* genes (Scherf et al., 2008). One third of the *var* gene repertoire is localized in the central part of the chromosome in a head to tail orientation (Gardner et al., 2002).
The *var* genes have a two exons arrangement separated by a conserved intron while exon I encodes the extracellular portion, exon II encodes the intracellular ATS (Figure 4B) (Gardner et al., 2002; Smith et al., 2000b). The chromosomal location and gene orientation are associated with 5´upstream sequences named *ups*. *var* genes belong to either one of four distinct *ups* classes A, B, C or E. *ups*A genes which are only found subtelomerically and are transcribed towards the telomere. *ups*C are always found internally while *ups*B can be found in both locations (Gardner et al., 2002; Kraemer and Smith, 2003; Lavstsen et al., 2003). The fourth group, *ups*E, is involved in *var2csa* expression. The overall organization is generally conserved in between parasites of genetically distinct background (Kraemer et al., 2007), and recombination tends to take place in between *var* genes belonging to the same *ups* group, therefore maintaining this genetic diversity (Bull et al., 2008; Gardner et al., 2002).
var2csa is an atypical *var* gene with a *5´upsE* sequence. The PfEMP1 encoded by this gene is the only one implicated in pregnancy-associated malaria and has the capacity to bind to CSA expressed in the placenta (Kyes et al., 2003; Salanti et al., 2003). As compared to proteins transcribed by other *var* genes, VAR2CSA displays significantly higher sequence conservation with 70-90% amino acid identity in between different isolates (Salanti et al., 2003; Trimnell et al., 2006).

1.5.4 *var* genes transcription and regulation

Of the 60 *var* genes present per haploid genome only one is selectively activated per parasite, with only one PfEMP1 expressed at a time on the parasite surface (Chen et al., 1998b; Scherf et al., 1998). Although two reports suggested multiple *var* gene transcription and PfEMP1 expression in *in vitro* cultures, the relevance of this phenomenon in patients has still not been investigated (Brolin et al., 2009; Joergensen et al., 2010). Parasites are able to switch *var* gene expression but also need to tightly regulate this process in order not to squander the entire repertoire. To date the exact molecular mechanisms underlying switching have not yet been identified. It appears

that each *var* gene possesses its own different switching rate, suggesting a hierarchical and non-random switching model (Horrocks et al., 2004). Additional data and mathematical modelling confirmed a non-random *var* switching, suggesting a balanced process between intrinsic switching and immune selection (Recker et al., 2011).
Whether external factors can influence the parasite switching pattern and rate seems logical but has not been proven yet. Few studies suggest that *in vitro* stimuli corresponding to starvation stress, high temperature or lactate levels can influence switching rate and pattern (Merrick et al., 2012; Rosenberg et al., 2009). Further, *upsA var* genes are frequently expressed in patients, with subsequent *in vitro* cultivation generating a random switching to non-A *var* genes (Bachmann et al., 2011; Blomqvist et al., 2010; Zhang et al., 2011b), suggesting a likely role for immune pressure in this phenomenon (Warimwe et al., 2009).

Regulation and silencing of *var* genes is a highly complex event that involves multiple regulatory-steps involving genetic and epigenetic regulation and subnuclear positioning. Two *var* genetics elements are of fundamental importance in gene regulation: the 5´ *ups* region and the intron can control gene silencing and activation (Dzikowski et al., 2006; Voss et al., 2006). The intron is responsible for silencing of the respective episomal *var* promoter and unpairing will cause gene activation and transgene transcription (Deitsch et al., 2001; Frank et al., 2006). This combined mechanism is of importance for *var* gene counting and activation of a single *var* gene at the time (Dzikowski et al., 2006; Voss et al., 2006). Recently, a sequence motif present in the 5´UTR of most of the *var* genes has been identified as critical for mutually exclusive expression, and its deletion revoked silencing of the corresponding gene (Brancucci et al., 2012).
Reversible histone modifications are pivotal in the process of *var* gene regulation (Freitas-Junior et al., 2005; Lopez-Rubio et al., 2007). Sir2A, a histone-deacetylase, is involved in gene silencing and Sir2A-knock out parasites display de-repression of *upsA* and *upsB var* genes (Duraisingh et al., 2005). Sir2B, a member of the same family, has a complementary repressing activity on *upsC var* genes (Tonkin et al., 2009). Overall histone deacetylation activity exerted by the Sir2 family plays an important role in *var* gene regulation, possibly allowing establishment of Histone 3 lysine 9 trimethylation, a silence heterochromatin mark enriched in repressed *var* genes promoter regions (Lopez-Rubio et al., 2007; 2009). Another recently uncovered silencing mechanism involves Histone 3 lysine 36 trimethylation regulated by *P. falciparum* variant silencing SET gene (PfSETvs). Upon PfSETvs knock-out the parasite loses the ability to repress *var* genes with simultaneous transcription of all the *var* genes and multiple PfEMP1 expression at the pRBC surface (Jiang et al., 2013). For a *var* gene to be activated, repressive histone marks need to be removed, rendering the gene permissive for transcription (Lopez-Rubio et al., 2007). Incorporation of particular histone variants, such as H2A.Z at the transcription start site has been associated with increased transcriptional activity (Bártfai et al., 2010; Petter et al., 2011). It appears that the apicomplexan specific H2B.Z histone variant has an important and specific role in *var* gene regulation: H2B.Z is exclusively present at the promoter of active *var* gene forming a double variant nucleosome with H2A.Z (Hoeijmakers et al., 2013; Petter et al., 2013).
The third control mechanism takes place via subnuclear positioning. Central *var* genes are tethered to the nuclear periphery where, together with subtelomeric *var* genes, they

cluster in perinuclear repressive centres (Lopez-Rubio et al., 2009; Ralph et al., 2005). For central *var* genes, the intron can interact with an actin recruiting complex and actin polymerization will then reposition central *var* genes at the periphery (Zhang et al., 2011a). In turn, one *var* gene will segregate from the perinuclear repressive centres and form a perinuclear expression site (Guizetti et al., 2013; Lopez-Rubio et al., 2009). The fine molecular details of the expression site remain elusive but evidence suggest that a common transcription site could be used by multiple variant surface antigens (Howitt et al., 2009).
It appears that all the mechanisms here described are tightly regulated in order to determine an impeccable *var* gene regulation, exclusion and switching. A final important element in *var* gene regulation is constituted by gene poising. Although *var* genes are transcribed only early in the life cycle, it has been demonstrated that parasites keep the same *var* gene transcribed after re-invasion (Guizetti and Scherf, 2013). In post-ring stages the activation mark Histone 3 lysine 4 bimethylation remains associated with the poised *var* gene (Lopez-Rubio et al., 2007) together with the H3K4 methyltransferase PfSET10. The two elements contribute together to the maintenance of the transcriptionally permissive chromatin environment, thus retaining epigenetic memory (Volz et al., 2012).

1.5.4.1 var genes and disease

When a parasite switches the PfEMP1 expressed on the RBC surface, its adhesion phenotype will most likely be altered as well. It is important to note that a number of studies determined that both *upsA* and *upsB/A var* genes are upregulated in children with severe disease while upregulation of *upsC* genes is more common in patients with milder disease (Avril et al., 2012; Bull et al., 2005; Claessens et al., 2012; Jensen et al., 2004; Kyriacou et al., 2006; Lavstsen et al., 2012). The cysteine content within a defined area of DBL1α co-varies according with *ups* sequence with *upsA var* genes carrying one or two cysteine while other *ups* containing three or more cysteine. Two cysteine/*upsA* proteins are commonly found in rosetting parasites and correlate with occurrence of severe malaria (Bull et al., 2005; Kirchgatter and Portillo, 2002; Kyriacou et al., 2006).
These results suggest that parasites expressing group A or B/A *var* genes have some selective advantage and are expressed first in a naïve host. The data obtained by analyzing parasite sequences are supported by the notion that individuals living in endemic areas acquire antibodies to PfEMP1 in an orderly manner, with antibodies (Abs) to group A and B/A proteins acquired first while Abs to PfEMP1 belonging to other groups are gradually acquired after repeated exposures (Cham et al., 2010; 2009) (further discussed in section *1.6.1*).

1.5.5 DBL domains structure and function

DBL domains are the building blocks of PfEMP1 (and some merozoites-adhesive antigens). The clarification of their structural features has been hampered for many years due to difficulties in recombinant protein expression, possibly due to the high number of formed disulphide bridges. The first crystal structure of a DBL domain was reported in 2005 by Tolia *et al.* (2005) with the structural description of the erythrocyte binding domain of EBA-175, RII. The following year the DBL-structure from *P.*

knowlesi was elucidated (Singh et al., 2006) showing a conserved common overall DBL-domain shape, despite the great sequence variation. Structural work on DBL domains derived directly from PfEMP1 molecules has proven to be challenging and so far the only structures that have been described derive from two distinct DBL domains from VAR2CSA, DBL3χ (Higgins, 2008; Singh et al., 2008) and DBL6ε (Gangnard et al., 2013; Khunrae et al., 2009), as well as from one DBL1α from rosetting PfEMP1 (Juillerat et al., 2011; Vigan-Womas et al., 2012). In the latter case, addition of the NTS to the molecule has proven necessary for correct folding and stability as well as crystallization of the DBL1α.

All DBL domains have an elongated shape, with 35-50% of the residues bundled in an α-helical core interspersed with extensive loop structures. The domain is canonically divided into three subdomains, namely subdomain-1 (SD1), subdomain-2 (SD2) and subdomain-3 (SD3) (Higgins, 2008; Juillerat et al., 2011; Khunrae et al., 2009; Singh et al., 2006; Tolia et al., 2005), with a recombination hotspot present between SD2 and SD3 (Rask et al., 2010).

SD1 displays the highest sequence variability between all known DBLs. It is mostly formed by random coils with two canonical intra-SD1 disulphide bridges (Higgins, 2008; Juillerat et al., 2011; Khunrae et al., 2009; Singh et al., 2006; Tolia et al., 2005).

SD2 forms a bundle consisting of four α-helices kept in place by one single conserved disulphide bridge, and interspersed with loop insertions of different lengths (Higgins, 2008; Juillerat et al., 2010; 2011; Khunrae et al., 2009; Singh et al., 2006; Tolia et al., 2005).

Two long α-helices separated by a long loop define the structurally conserved SD3. A third α-helix, distal from the loop is also conserved among disting DBLs. The number of disulphide bridges is variable, with a minimum of four in order to hold the global shape. The first disulphide bridge is present at the beginning of the first helix, a second and third at the extremities of the loop while the fourth is distal to the loop, holding the two helices together (Higgins, 2008; Juillerat et al., 2011; Khunrae et al., 2009; Singh et al., 2006; Tolia et al., 2005).

Only one NTS structure is available to date: this section of PfEMP1 includes one long and two short α-helices. One short β-hairpin forms a pincer structure holding together one of the NTS helices with helix-7 of DBLα. NTS is an integral part of the DBLα domain. Indeed, despite cleavage of the NTS from the DBL core, the two molecules remain associated and full separation is possible only under denaturing conditions (Juillerat et al., 2011; Vigan-Womas et al., 2012).

1.5.5.1 DBLβ-C2:ICAM1

The DBLβ-C2 domain from group B and C PfEMP1 is the ICAM1 binding partner on the pRBC surface (Chattopadhyay et al., 2004; Howell et al., 2008). A recent report underpinned these findings through the use of surface plasmon resonance (SPR), indicating that the affinity of the interaction between the domain and the receptor is in the low nanomolar range, nearly identical to the one of ICAM-1 with full length PfEMP1 IT4var13 (Brown et al., 2013).

The low-resolution structure of PfEMP1-ICAM1 complex has been elucidated using small angle X-ray scattering (SAXS). Cytoadhesive PfEMP1 has a “pearls on a string” conformation with all individual domains fully solvent accessible at the pRBC surface. Interestingly the DBLβ domain protrudes from the protein core, allowing for

interactions with the tip of ICAM1 (Brown et al., 2013).
The important residues for this interaction have been identified by site directed mutagenesis, and confirmed by the SAXS co-structure, and have been mapped to the SD2 region, the same involved in glycan binding in EBA-175(Brown et al., 2013; Howell et al., 2008; Tolia et al., 2005).

1.5.5.2 PfEMP1(VAR2CSA):CSA

VAR2CSA interaction with CSA is the hiertho best characterized. There is clear evidence that the *var2csa* gene is highly up regulated in parasites from infected placenta (Duffy et al., 2006; Tuikue Ndam et al., 2005). VAR2CSA has a particular structure with no NTS-DBLα domain but three DBLχ and three DBLε domains. Early research focusing on individual domains identified several of these as possible binders to CSA (Avril et al., 2006). In addition, the crystal structure of DBL3χ was determined in complex with CSA, revealing the exact residues involved in this interaction (Singh et al., 2008). Later studies casted doubts on the specificity and selectivity of individual domains binding to CSA for this specific PfEMP1 (Khunrae et al., 2009; Resende et al., 2009). The expression of full-length VAR2CSA protein and the solution of its structure by SAXS suggested that this particular PfEMP1 variant assumes a compact, spherical conformation (Clausen et al., 2012; Srivastava et al., 2010). The full length protein binds to CSA with nanomolar affinity, a value approximately thousand times lower compared to the binding of single domains (Khunrae et al., 2010). Further studies that generated a series of truncated versions of VAR2CSA finally identified the DBL2χ domain as the minimal binding region. However it should be noted that high affinity interactions are only obtained in the presence of the inter-domain regions flanking the DBL2χ domain (Clausen et al., 2012; Srivastava et al., 2011). The combination of VAR2CSA structural data with binding data suggests an atypical, multivalent protein-sugar interaction where multiple binding sites and non-ionic bonds are required for high affinity binding (Clausen et al., 2012).

1.5.5.3 DBLα:rosetting ligands

DBLα is the only domain that has so far been identified as responsible for rosetting. It is the most distal PfEMP1-domain from the pRBC surface and this might provide an advantage for adhesion to non-parasitized RBC. It is the domain that has the highest degree of conservation among DBLs and contains two hyper-conserved sequences present in all DBLα sequences (Flick and Chen, 2004; Rask et al., 2010; Smith et al., 2000b). Bull and colleagues, using conserved tags, identified specific sequence signatures that are associated with rosetting phenotype. In particular sequences with one or two cysteine in between those tags belong to groups PolV 1-3 (DBLα1), while sequences with three, four or five cysteine belong to groups PolV 4-6 (Bull et al., 2005). This is of interest because nearly all group A DBL1α1 have two cysteine (Trimnell et al., 2006), a feature associated with elevated rosetting and severe malaria (Bull et al., 2005; Kirchgatter and Portillo, 2002).
Three DBL1α rosetting domains have been well characterized for their receptor specificities. DBL1α from the parasite R29/IT4var9, expressed on COS-7 cells, binds to CR1 expressed on RBCs, a binding that can be competed out by soluble CR1 (Rowe et al., 1997). The central DBL region, localized between SD2 and SD3 (aa 133-366) is

responsible for binding (Mayor et al., 2005). However, it has not been possible to detect any binding between soluble CR1 and DBL1α of R29 by using SPR (Tetteh-Quarcoo et al., 2012). The recombinant NTS-DBL1α-R29 is able to disrupt rosettes formed by the homologous parasite strain (Mayor et al., 2009).
The NTS-DBL1α expressed by the highly adhesive parasite FCR3S1.2 is encoded by the IT4var60 gene (Albrecht et al., 2011). This parasite binds to multiple receptors, including BgA and HS (Barragan et al., 2000a; 2000b) however the residues involved in binding have not been elucidated and will be discussed in **Paper V**.
Finally the third and best characterized rosette-mediating DBLα belongs to the PAvarO parasite (Vigan-Womas et al., 2008). Two crystal structures have been reported for this protein, the second comprising the entire head structure (NTS-DBL1α-CIDRγ domains) (Juillerat et al., 2011; Vigan-Womas et al., 2012). Although PAvarO parasite rosettes are heparin sensitive, HS is not the receptor for this parasite variant (Juillerat et al., 2010). Heparin binds to the DBL domain in the NTS region as well as in SD3 (Juillerat et al., 2011). When analysing the head structure, BgA was identified as the rosetting receptor (Vigan-Womas et al., 2012). The BgA binding site localizes in SD2, on the opposite face of the molecule as compared to the heparin binding site (Vigan-Womas et al., 2012). The mechanisms behind heparin-mediated rosette inhibition are still elusive. Heparin-induced multimerization has been suggested but further proof is needed to support this hypothesis (Juillerat et al., 2011). The multiple binding of NTS-DBL1α of PAvarO to both A and O RBCs is intriguing and of importance to understand malaria pathogenesis (Vigan-Womas et al., 2012). Whether this feature is shared by other parasite strains needs to be investigated.

1.5.6 Other variant surface antigens

1.5.6.1 rif/RIFIN

The variable RIFIN polypetides have a molecular weight ranging from 30 to 50 kDa (Fernandez et al., 1999; Kyes et al., 1999). The proteins are encoded by 150-200 *rif* (repetitive interspersed family) genes per genome, with 149 copies in the 3D7 genome (Gardner et al., 2002) located subtelomerically in conjunction with *stevor* and *var* genes. The genes are encoded by two exons and can be subdivided in two groups (A and B) depending on the presence of a 25-aminoacid "indel" sequence, localized approximately 100 amino acids downstream towards the N-terminal (Joannin et al., 2008). A and B RIFIN appear to have distinct subcellular localizations with A-RIFINs expressed during trophozoite and schizont stages. A-RIFINs and PfEMP1 co-localize in the Maurer´s clefts, both in laboratory and in patient parasite isolates, and they are possibly co-transported to the pRBCs surface (Bachmann et al., 2012; Haeggström et al., 2007; 2004; Petter et al., 2007). Further, A-RIFINs localize to the merozoite apex (Petter et al., 2007). In contrast, B RIFINs are localized intracellularly during the life cycle and emerge only later in association to merozoites (Mwakalinga et al., 2012; Petter et al., 2007). B RIFINs appear also to be expressed on gametocyte surface (Mwakalinga et al., 2012).
Logically the high number of variants encoded by the *rif* genes in the genome suggests these proteins to be exposed on the erythrocytes surface and to be subjected to immune pressure. Further, it has been shown that plasma from semi-immune adults recognize recombinant RIFIN proteins, while recognition in children´s sera was much lower

(Abdel-Latif et al., 2002). Moreover presence of anti-RIFIN Abs was higher in asymptomatic children compared to severe malaria cases and positively correlated with parasite clearance (Abdel-Latif et al., 2003). Their function remains to be elucidated.

1.5.6.2 stevor/STEVOR

The sub-telomeric variable open reading frame (STEVOR) is a family of proteins of around 30-40 kDa (Cheng et al., 1998). About 30-40 *stevor* genes are present per genome and localize mostly in sub-telomeric region, with few of them being centromeric (Cheng et al., 1998; Gardner et al., 2002).
STEVORs seem to be expressed during a number of parasite developmental stages including trophozoites, schizonts (Kaviratne et al., 2002; Niang et al., 2009), merozoites (Blythe et al., 2008; Khattab and Meri, 2011; Khattab et al., 2008) and gametocytes (Tibúrcio et al., 2012). They localize in Maurer´s clefts (Kaviratne et al., 2002), are present on pRBCs surface (Niang et al., 2009) and are highly expressed in patients isolates (Bachmann et al., 2012; Blythe et al., 2008).
Their expression during trophozoite and schizont stages impacts the RBC membrane rigidity, making the cells less deformable and suggesting a possible role in microvasculature sequestration (Sanyal et al., 2012). Moreover the same function is exerted in early stage gametocytes (Tibúrcio et al., 2012).
Finally, their association with merozoites also suggests a possible additional involvement in invasion (Blythe et al., 2008; Khattab and Meri, 2011; Khattab et al., 2008).

1.5.6.3 surf/SURFIN

Approximately 10 subtelomeric *surf* genes per genome encode for the same number of high molecular weight SURFINs proteins (Gardner et al., 2002). SURFINs are expressed both on the surface of schizonts as well as on merozoites (Mphande et al., 2008; Winter et al., 2005). Their function still remains elusive.

1.6 HUMORAL IMMUNITY TO *PLASMODIUM FALCIPARUM* MALARIA

Acquisition of immunity to *P. falciparum* malaria is a complex mechanism that has not yet been fully elucidated. Individuals living in endemic areas gradually acquire immunity, at a rate depending on the intensity of transmission (Figure 5) (Doolan et al., 2009; Langhorne et al., 2008). Children under 3 months of age are protected against malaria but the mechanisms underlying this protection are unclear, with maternal IgG as well as IgA and lactoferrin possibly playing an important role (Fouda et al., 2006; Kassim et al., 2000; Riley et al., 2000). At 3 to 4 month of age infants become vulnerable to severe disease, a susceptibility that lasts until the child is 2 to 5 years of age, depending on the transmission intensity. Mild malaria can still occur in young children until they reach adolescence, where they are virtually fully protected from symptomatic infections, although sterile immunity towards parasites is probably never achieved (Langhorne et al., 2008). In addition, interruption of exposure may lead to loss of protection, implying that constant parasite exposure is needed to maintain protection (Doolan et al., 2009). In areas of high malaria transmission immunity to

severe disease, but not to cerebral malaria, is reached just after one or two infections (Gupta et al., 1999).
Antibodies to blood stage antigens seem to be the key players in acquisition and maintenance of protection in natural infection settings (Langhorne et al., 2008), as also confirmed by data from mouse models, where parasite clearance is dependent on B cells and antibodies (Langhorne et al., 1998). Early experiments, that involved passive transfer of IgG from immune adults to non-immune children demonstrated that IgG have a central role in the protection from severe disease (Cohen et al., 1961; McGregor et al., 1963). Mechanisms of protection are many, and include blockade of merozoite invasion of RBCs (Blackman et al., 1990), binding to parasite proteins exposed on RBCs surface thus inhibiting adhesion and facilitating clearance (Baruch et al., 2002; Bull et al., 1998; Moll et al., 2007) and Ab-dependent cellular killing by cytophilic Abs (IgG1 and IgG3) (Bouharoun-Tayoun et al., 1995).

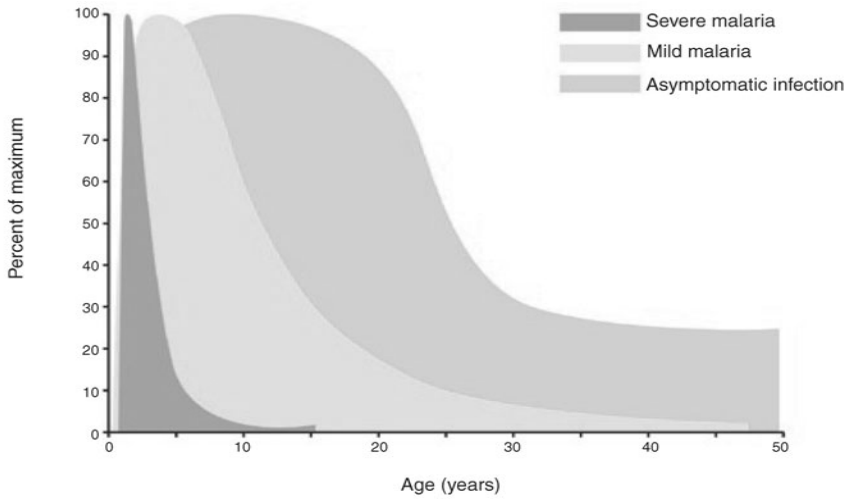

Figure 5. Acquisition of immunity to malaria by individuals living in endemic areas. (Adopted from Langhorne, 2008 and reproduced with permission from Nature Publishing Group).

Despite the constant parasite exposure, it is interesting to note that the Ab response is usually extremely short lived, a fact that could be due to a defect in establishing B-cell immunological memory (Portugal et al., 2013). Generation of malaria-specific memory B cells is achieved in a stepwise manner, following a number of exposure to the parasite, a result that is opposite to classical cases, including tetanus or measles, where memory B-cells are acquired after the first infection (Weiss et al., 2010). Further, the constant activation of B-cells by malarial antigens and parasite exposure might be a cause for B-cell exhaustion with inefficient acquisition and rapid-loss of specific B-cells (Portugal et al., 2013; Weiss et al., 2011). Indeed it seems that malaria infection leads to development and accumulation of atypical memory B cells, whose ability to generate potent Abs remains unclear (Muellenbeck et al., 2013; Weiss et al., 2011; 2009).

1.6.1 Antibody response to PfEMP1

PfEMP1 is one of the major targets of the Ab response to the parasite during the erythrocytic stage (Bull et al., 1998; Crompton et al., 2010; Dodoo et al., 2001; Nielsen

et al., 2002; Ofori et al., 2002) with most of the Ab recognition of pRBCs being abolished in PfEMP1-KO parasites (Chan et al., 2012). Anti-PfEMP1 Abs are acquired from the first *P. falciparum* infection (Elliott et al., 2007; Turner et al., 2011) and Abs to specific PfEMP1 variants are acquired in an ordered manner, first to group A and subsequently to other groups (Cham et al., 2009; 2010). This finding supports the hypothesis that immunity to severe malaria-associated variants is acquired first and during subsequent infections the parasite is forced to switch to less virulent, less adhesive variants that do not cause severe disease.

What is still generally unclear is the nature of the PfEMP1-specific Abs and in particular whether protection is achieved by the acquisition of a pool of monospecific Abs or rather by few cross-reactive antibodies. Several reports from monkey experiments suggest a superior protection against homologous parasite strains rather than heterologous (Fandeur et al., 1995; Jones et al., 2000; Udeinya et al., 1983). This is indeed not surprising and if broad cross-reactivity exists it would be possibly achieved after few infections with genetically distinct parasites. Further, in naive adults moving to endemic areas acquisition of immunity to severe disease is rather fast (Baird et al., 2003) and intuitively the antigenic repertoire of the parasite is much larger than the number of infections that an adult could experience throughout his life (Doolan et al., 2009). However there is currently no definite answer to which of the two mechanisms is predominant in malaria infection.
An evidence in favour of the presence of cross-reactive Abs in the population is the ability of sera derived from semi-immune adults to recognize parasites isolates, to which they have probably never been exposed before (Elliott et al., 2007; Ofori et al., 2002; Vigan-Womas et al., 2008; 2010 and **Paper IV**). Additional strong evidence for the existence of cross-reactive Abs comes from PAM, where pregnant women become sick during their first pregnancy but do not develop disease in subsequent pregnancies, a protection that is associated with the generation of anti-PfEMP1-VAR2CSA Abs (Salanti et al., 2004; Staalsoe et al., 2004). Recent studies in the HIV field suggest that a combination of just few broadly neutralizing Abs are sufficient to fully control virus infection (Klein et al., 2012). Further it has been shown that for seasonal influenza re-stimulation of memory B-cells induces maturation and generation of potent cross-neutralizing Abs against conserved epitopes (Wrammert et al., 2011). Whether these mechanisms are common to *Plasmodium* infection and PfEMP1 immunity is currently unknown.

1.6.1.1 Antibodies to VAR2CSA induced by immunization

In recent years a great amount of studies have been performed on vaccination-induced antibodies, and particularly on the highly conserved VAR2CSA protein.
Various VAR2CSA domains can induce antibodies that block *P. falciparum* adhesion to CSA (Avril et al., 2011a; Bigey et al., 2011; Magistrado et al., 2011; Nielsen et al., 2009; Oleinikov et al., 2008; Pinto et al., 2011; Salanti et al., 2010), a characteristic that is desirable and similar to what is seen in natural infections. The higher sequence conservation and conserved binding characteristics of this particular PfEMP1 makes the experimental generation of cross-reactive Abs relatively easy (Avril et al., 2010; 2008; Bigey et al., 2011; Magistrado et al., 2011; Saveria et al., 2013). Intriguingly, immunization with full length VAR2CSA elicits strain-transcending Abs that

surprisingly cannot cross-inhibit adhesion of heterologous parasites (Avril et al., 2011b), a characteristic that is rather common upon single domain immunization (Avril et al., 2008; 2010; Fernandez et al., 2008; Magistrado et al., 2011; Nielsen et al., 2009; Saveria et al., 2013). Discordant reports suggest that different domains have different capability in inducing cross-reactive Abs. However there is also a general consensus that C-terminal domains DBL4-5-6ε are the most potent (Avril et al., 2011a; Fernandez et al., 2008; 2010; Magistrado et al., 2011; Nielsen et al., 2009; Saveria et al., 2013). As discussed in paragraph *1.4.3.1*, the VAR2CSA binding site lies in the N-terminal part of the molecule. The mechanisms by which the described antibodies exert their adhesion-inhibitory action are not fully elucidated. The close proximity of the domains in the full-length protein structure suggests that inhibition could depend on direct steric hindrance, but conformational changes upon Ab binding cannot also be excluded (Clausen et al., 2012). Mapping of important epitopes targeted by inhibitory Abs has been attempted by human serum depletion on parasites (Dahlbäck et al., 2006) as well as by comparison of non-inhibitory versus inhibitory Ab reactivity upon immunization (Ditlev et al., 2012; Pinto et al., 2011). However monoclonal Abs (mAbs) could give more biologically relevant insights: few mAbs have been generated so far against VAR2CSA (Avril et al., 2006; Barfod et al., 2007), with a panel of them being IgM and their epitope specificity not tested (Avril et al., 2006). A number of human mAbs has been isolated for their specific recognition of VAR2CSA: for one of them (PAM 8.1), which is moderately cross-inhibitory, the epitope has been mapped to the loop localized in SD3 of DBL3χ (Barfod et al., 2007).

1.6.1.2 Antibodies to rosetting DBL domains induced by immunization

The generation of cross-reactive antibodies upon immunization with rosetting domains has proven to be more challenging compared to VAR2CSA domains. Immunization of monkeys and rats with Semiliki-Forest virus particles encoding DBL1α domains (Chen et al., 2004; Moll et al., 2007) demonstrated that immunization with single domains could generate antibodies that inhibit *in vivo* sequestration of heterologous pRBCs and immune-sera could disrupt heterologous parasites rosettes (Moll et al., 2007).
Recently, specific and potent anti-rosetting Abs have been generated towards few rosetting parasite strains upon immunization with recombinant proteins produced in *E. coli* (Ghumra et al., 2011; Vigan-Womas et al., 2008; 2011; **Paper I**). While all reports showing that the NTS-DBL1α domain is able to generate antibodies with anti-rosetting activity on the homologous parasite strain, it appears that for the R29/IT4var9 strain multiple PfEMP1-domains are able to elicit rosette disruptive Abs (Ghumra et al., 2011). Work from Vigan-Womas *et al.* (2011) suggests that natural immunity is acquired through a collection of Abs to non-overlapping serotypes. However, subsequent work challenged this hypothesis, showing that upon immunization with recombinant protein strain-transcending antibodies could be obtained (Ghumra et al., 2012). Ghumra *et al.* (2012) show that Abs raised against NTS-DBL1α, derived from parasites with a specific virulence-associated phenotype (IgM-positive rosetting), are able to cross-recognize other laboratory strains and patients isolates possessing the same phenotype and, in most cases, disrupt their rosettes. This could suggest that strain-transcending antibodies are directed towards restricted groups of parasites presenting the same virulence phenotype with natural immunity acquired through a piecemeal acquisition of phenotype-specific antibodies.

2 SCOPE OF THE THESIS

The overall aim of this thesis was to better understand the rosetting phenomenon in *Plasmodium falciparum*. We investigated the NTS-DBL1α domain of the variable surface antigen PfEMP1 and consequently improved the knowledge of specificity and mode of action of antibodies induced by vaccination and by natural infections. We also dissected the biochemical properties of the NTS-DBL1α domain itself.

Specific Aims

I. To investigate the epitopes recognized by rosette disruptive antibodies and to define immunodominant regions of the NTS-DBL1α molecule.

II. To understand the diversity of antibody responses in animals immunized with NTS-DBL1α domain and define their levels of cross-reactivity.

III. To identify surface exposed sequence motifs, in the DBL1α, shared by distinct PfEMP1 expressed by different parasite strains.

IV. To study the naturally acquired antibody response to the NTS-DBL1α domain and its association with anti-rosetting activity.

V. To determine the RBC binding site of one NTS-DBL1α variant and define the protein-antibody interaction.

3 EXPERIMENTAL PROCEDURES

All the materials and methods are described in great detail in each respective study (**Papers I to V).** Methods of particular importance for the understanding of the thesis will be highlighted in this chapter.

3.1 PARASITE *IN VITRO* CULTURE

Parasites used in these studies were cultivated *in vitro* in O+ red blood cells, 5% haematocrit and 10% A+ serum in buffered malaria culture medium. Parasites were cultivated in a gas mixture consisting of 90% NO_2, 5% O_2 and 5% CO_2 in shaking incubation. pRBCs were synchronized using 5% sorbitol treatment for 10 minutes.
The rosetting phenotype was preserved either by Ficoll selection or by mAb selection. For mAb selection pRBCs were incubated with selected antibodies at 25μg/ml and subsequently purified using anti-mouse MicroBeads on an LS MACS column.

3.2 RECOMBINANT PROTEIN EXPRESSION AND PURIFICATION

Recombinant proteins were expressed in *E. coli* and either purified from inclusion bodies or from the soluble fraction.
For purification of inclusion bodies *E. coli* BL21 (DE3) strain was used: bacteria were transformed, with sequence cloned into pQE60/70 expression vector, and grown till OD=0.8 at 37°C. After induction with 0.1mM IPTG for 3 hours at 37°C the cells were lysed by sonication and crude inclusion bodies were pelleted by centrifugation at 12,000*g* for 30 minutes. The pellet was solubilized in denaturing solution, containing 6M Guanidine HCl, overnight at 4°C. After centrifugation at 50,000*g* for 1 hour the solubilized proteins were refolded by rapid dilution method: proteins were added dropwise to ice-cold refolding solution (200mM Tris-HCl pH 8, 10mM EDTA, 0.6M arginine, 6.5mM cysteamine and 3.7mM cystamine) to a final concentration of 0.2mg/ml. Refolding was allowed to proceed for 36 hours at 4°C. Excess EDTA was removed by dialysis and protein concentrated before purification.
For soluble protein purification *E. coli* Shuffle T7 express strain was used: bacteria were transformed, with recoded sequence cloned into pJ414 expression vector, and grown till OD=0.6 at 30°C. After induction with 0.4mM IPTG for 20 hours at 16°C the cells were resuspended in sucrose buffer, subjected to osmotic shock and subsequently lysed by sonication. The soluble fraction, containing the recombinant protein, was separate by centrifugation at 50,000*g* for 20 minutes and used for purification.
A two-step purification was carried out by Immobilized Metal Affinity Chromatography (IMAC), on Nickel or Talon columns, followed by size exclusion chromatography on HiLoad 16/60 Superdex 75pg column.

3.3 MONOCLONAL ANTIBODY PRODUCTION IN MICE

mAbs were produced through a collaboration with the European Molecular Biology Laboratory (EMBL) monoclonal antibodies core facility (MACF) according to their standard routines (De Masi et al., 2005). Two BALB/c mice were immunized with 50 μg of each protein three times at one-month interval, using Alum adjuvant. Mice were

bled 10 days after each immunization and antibody titres tested by ELISA. Upon positive recognition of antigen in ELISA, spleen cells were mechanically disrupted into single cell suspension and fused with SP2 myeloma cells in a ratio of 5:1. Post-fusion cells were transferred into 96 wells plates, monoclonality was achieved by limiting dilution. On day 11 after fusion the supernatants were tested for recombinant protein recognition by using an antigen microarray.

3.4 POLYCLONAL ANTIBODY PRODUCTION

Polyclonal antibodies were produced in goat and rats by Agrisera, Sweden. Immunization was carried out intramuscularly with 200µg of protein injected four times at one-month interval. For the first immunization the protein was emulsified in Freund´s complete adjuvant while Freund´s incomplete was used for the other three immunizations. Recognition of the recombinant proteins was verified by ELISA and western blot and, if positive, animals were bled two weeks after the last immunization.

3.5 RED BLOOD CELL BINDING ASSAY

Red blood cell binding assay was carried out by flow cytometry. Different concentrations of purified recombinant proteins were incubated with RBCs (approximately $2x10^7$ cells per well) in PBSF (PBS / 2% FCS) for 30 minutes. After three washes with PBSF, mouse anti-his antibody (Qiagen) was added to each well at a 1:200 dilution for 30 minutes. Three washes were followed by 30 minutes incubation with anti-mouse Alexa 488 conjugated. Washed cells were resuspended in 200µl PBSF and analysed by flow cytometry on FACScan (BD Bioscience) where 100000 cells were counted per sample. Each sample was run in duplicates. Data were analysed using FlowJo (TreeStar).

3.6 ROSETTE DISRUPTION ASSAY

Synchronized trophozoites 28-34 hours post invasion with a rosetting rate of 60% or above were used for the rosette disruption assays. 45µl of parasite culture were transferred per well in a 96 well plate (using a pipette tip widely open in order to avoid mechanical disruption) and 5µl of antibody solution, at different concentration, added to each well. Non-immune IgG, control mAb or pre-immune sera were used as control in all experiments. After one hour incubation at room temperature, acridine orange was added to the suspension and a drop of parasite suspension was placed on a microscopy slide. The rosetting rate was counted under a fluorescent microscope with at least 20 fields and 200 pRBCs per slide. Slides were moved diagonally from one corner to the opposite. The rosetting rate was expressed as a percentage compared to the rosetting rate of pRBCs incubated with PBS only.

3.7 FLOW CYTOMETRY ANALYSIS

For flow cytometry analysis, synchronized trophozoites 24-30 hours post invasion were used. pRBCs were blocked for 30 minutes in PBSF and thereafter incubated with antibodies/serum for 30 minutes at room temperature in a volume of 50µl. After three washes with PBSF parasites were resuspended in 50µl of PBSF containing Alexa-488

conjugated secondary antibody for the relevant species. For nuclear staining ethidium bromide at final concentration on 2.5µg/ml was added and cells finally washed three times and resuspended in 200µl PBSF. Cell acquisition was done using FACScan (BD Bioscience) where 5000 infected cells were counted per sample. Each sample was run in duplicates. Data were analysed using FlowJo (TreeStar). For **Paper III**, adjusted MFI ratio was used to compare samples, in order to take day-to-day variability of flow cytometry assay into consideration. The surface reactivity was expressed as geometric mean fluorescent intensity (MFI) and adjusted according to pre immune reactivity and reactivity on non parasitized RBCs, according to the following formula: $(pRBC_{immune}/pRBC_{non\text{-}immune})$- $(RBC_{immune}/RBC_{non\text{-}immune})$.

3.8 PEPTIDE ARRAY

For detailed mapping of antibody binding sites, peptide-arrays of overlapping peptides were produced. The peptides were bound chemoselectively to the microarray surface by coupling an active amine of the peptide to an epoxy-group on the slide surface. The peptide microarrays were manufactured by JPT (JPT Peptide Technologies, Berlin, Germany) and each slide contained three identical sub-arrays. The arrays were holding NTS-DBL-domains from long-term cultivated parasites as well as from Ugandan isolates. The peptides were 15 amino acid long, with an overlap of 11 amino acids. The slides were incubated for 16 hours with relevant antibody at 4^{o}C in a humid chamber in PBS buffer containing 3% of FCS and 0.5% of Tween (T-PBS). After five washes the slides were incubated with 1:500 Cy5-conjugated secondary antibody for the relevant species (Jackson ImmunoResearch) for two hours at room temperature in a humid chamber. Following three washes the microarrays slides were scanned at wavelength of 635nm using a GenePix 4000B microarray scanner (Axon Instruments, CA, USA). The images were analysed using GenePixPro 7.0 software.
Following automated analysis and flagging of the spots, visual inspection of the images from the individual subarrays was carried out in order to evaluate responses before the analysis. For a measure of the strength of the response, we chose the ratio of median foreground to background (on a log scale). This response index was computed for all spots with a background greater than zero, and all spots with zero background were excluded. Any peptide with a high response on slides incubated only with buffer and the secondary Cy5-labeled was considered false positive and discarded for analysis. After normalizing all valid peptide responses on the buffer slides using the same linear model as for the negative controls, the cutoff was determined for the definition of a false-positive event. The data presented represent the average of three subarrays.

3.9 ETHICAL APPROVALS

Ethical approvals for human participation in the studies included in this thesis were obtained both from ethical research committee at Karolinska Institutet as well as in the country where the studies were performed. Informed consent was obtained from the parents or guardians of the children.
Ethical approvals for animal immunizations were obtained from the animal ethical committee in Sweden.

4 RESULTS AND DISCUSSION

The results, interpretations and discussion of the five papers included in this thesis are summarized below. Results are also discussed in light of more recent discoveries.

4.1 PAPER I

Epitopes recognized by anti-rosetting monoclonal antibodies are localized in Subdomain-3 of NTS-DBL1α.

The molecular mechanisms underlying rosette inhibition are still unclear. Identifying the targets of rosette-disruptive antibodies will be of importance for the rational design of specific drugs and/or for a vaccine targeting rosette-formation that could protect against severe malaria.
We approached the problem by generating a large set of monoclonal and polyclonal antibodies directed against NTS-DBL1α domains derived from laboratory rosetting parasite strains (FCR3S1.2^{IT4var60}, R29^{IT4var9} and PAvarO). Mouse mAbs were produced in collaboration with EMBL by fusion of spleen cells with SP2.0 cells and individual hybrydoma clones were tested for protein recognition by high throughput ELISA. Goats were immunized and sera were tested by ELISA and FACS. Each goat yielded approximately one litre of serum from which IgG were purified.
The generated antibodies were pivotal for the rest of the study: all the reagents were verified for their ability to recognize the pRBC surface by FACS and rosette disruption assays. In particular the mAbs could be divided into three functional groups that allowed us to dissect distinct regions on the NTS-DBL1α molecule.
The first group comprised the mAbs that were surface reactive and rosette disruptive, the second group in the antibodies that could recognize the pRBC surface but did not disrupt rosettes and the third group included mAbs neither recognizing the pRBC surface nor disrupting rosettes, but were still positive in ELISA.
With the use of a peptide array, covering the whole NTS-DBL1α domain, we were able to map the epitopes recognized by different mAbs within the functional groups. Our findings showed that all the mAbs from the first group, which recognized the pRBC surface and disrupted rosettes in the parasite lines FCR3S1.2 and R29, mapped to an epitope localized in the loop structure connecting α-helices 6 and 7 in subdomain 3 of DBL1α, suggesting this to be an important immunodominant site of the molecule. Further, the other two groups of mAbs allowed us to discriminate and identify which regions of NTS-DBL1α are surface exposed and which are instead buried in the full length PfEMP1.

For further analysis and to underline the importance of SD3 in rosetting of the FCR3S1.2 parasite we took advantage of the generated IgG from goats. The peptide array revealed that while most of the reactivity was directed against NTS and subdomain 1 only few reactivity peaks were detected in subdomains 2 and 3 (one being the peptide corresponding to the mAb epitope, localized in the SD3-loop). In order to verify the relative importance of antibodies toward this epitope, we absorbed out the reactivity directed to this peptide and tested the "depleted" IgG for surface reactivity and rosette disruption. The ability of the depleted IgG to recognize the pRBCs surface

was intact but the capacity to disrupt rosettes was reduced by approximately 80%. Further, immunization with a single subdomain 3 from IT4var60, but not subdomain 1 or 2, could generate surface and rosette active antibodies.
To determine whether antibody response towards SD3-loop is common during natural infections, we investigated the recognition of peptides, corresponding to the SD3 loop region of the three parasites strains studied herein, by sera derived from individuals residing in endemic areas. Sera from children from Uganda generally showed a high recognition of all three peptides, as detected by ELISA, and the levels were correlated. The data presented suggest the SD3-loop epitope to be a common target of antibodies in individuals living in areas of high malaria endemicity.
After identifying the SD3 loop as critical target for rosette disrupting antibodies, we analysed several NTS-DBL1α sequences for phylogenetic grouping according to SD3 sequences. Unfortunately with the tools available to date and the lack of conservation downstream of subdomain 2 it was difficult to extensively study sequences from isolates directly collected from patients and relate them to the ability to form rosettes and disease status. It is known that the presence of two cysteine in SD2 is associated with rosetting phenotype and severe disease (Bull et al., 2005; Kirchgatter and Portillo, 2002) and our grouping, based on SD3, mirrored this division. Our data suggest the presence of sequence determinants in SD3 possibly associated with disease severity.

Our study supports a role of SD3 as a major target of anti-rosetting antibody response and reveals the molecular details behind the strain specific anti-rosetting response. Polyclonal antibodies offer little information about the targeted epitopes and whether the antibodies directed to the same epitopes are responsible for the cross-recognition of distinct parasites. In this study we identified a common site recognized by mAbs in different parasite strains. A chimeric vaccine comprising this minimal region derived from several parasite strains could induce broadly reactive rosette disruptive antibodies. Identifying the area of antibody binding is of importance to better understand immune responses and *P. falciparum* rosetting in general, and this is the first study reporting detailed epitope mapping of PfEMP1. Previous reports on other parasite strains identified the heparin-binding site in subdomain 1 and BgA binding site in subdomain 2. Further in **Paper V** we report the RBC binding site for IT4var60 to be localized in subdomain 2.
The discordance of the sites identified in this paper as epitope and in other studies as receptor binding is not fully surprising. Other pathogens such as HIV are well known to focus immune responses towards polymorphic sites in order to avoid Ab binding at the receptor site (Karlsson Hedestam et al., 2008). Further, results similar to the one obtained here have been reported for *P. vivax* Duffy binding protein (DBP). While the binding site localizes on SD2 of the molecule (Batchelor et al., 2011; Chootong et al., 2010) only antibodies towards SD3 are able to disrupt parasite binding and recognize different parasites (Siddiqui et al., 2012).
The corresponding SD3 loop of the DBL3χ domain for the placental malaria PfEMP1, VAR2CSA is also target of human antibodies (Barfod et al., 2007). Furthermore SD3 of ICAM-binding DBLβ mediates receptor binding and is target of adhesion inhibitory antibodies (Bengtsson et al., 2013). To better understand the mode of inhibition of the mAbs hereby studied a co-crystal structure together with the NTS-DBL1α domain would be needed. This would reveal the entire conformational epitope of the antibody and important structural information regarding the interaction. The cleft formed by SD2

and SD3 comprises multiple positively charged residues (lysine and arginine) and the receptor-binding could be a multi-step event with SD3 being responsible for the first contact and SD2 for the final high-affinity interaction with the receptor.
Altogether the data reveal a great complexity that we just now start to unravel. It appears that SD3 is a common target for biologically active antibodies in a number of Duffy Binding proteins.

4.2 PAPER II

High antibody titres towards NTS-DBL1α can be consistently induced in different animal species. Cross-reactivity is common in ELISA but rare on live pRBCs.

In **Paper I** we have shown that rosette inhibitory antibodies can be raised against different parasite variants, however it was unclear weather that could be achieved consistently in different animal species: a feature that is of interest for the possible development of a vaccine that includes the NTS-DBL1α domain. In order to answer this question we immunized rats and goats with three distinct NTS-DBL1α domains derived from different rosetting laboratory parasite strains.
The recombinant NTS-DBL1α proteins were expressed in *E. coli* and while the PAvarO and R29 constructs were purified from inclusion bodies, the IT4var60 was expressed as soluble protein. All the generated proteins were pure and highly homogeneous thus suitable for immunization studies. Interestingly, we found that all the constructs could induce high titres of antibodies irrespectively of the animal species. Further, all induced antibodies were able to recognize the surface of homologous pRBCs. In contrast a previous study has shown that different domains from the pregnancy malaria vaccine candidate VAR2CSA (Pinto et al., 2011) were not able to consistently generate surface- and ELISA- reactive antibodies in different animal species. Indeed most of the VAR2CSA domains failed to generate efficient surface reactive antibodies.

The second aspect we addressed in this study was whether we could induce antibodies that are able to cross-react, both in ELISA as well as on the live pRBC surface, and define the cross-reactive epitopes on a linear peptide array. In our experiment we could detect extensive cross-reactivity by ELISA towards heterologous NTS-DBL1α domains, cross reactivity that was further confirmed by peptide array where several of the peptides from heterologous proteins were recognized by different sera. However, most of those peptides were shown to be not-surface exposed according to the available crystal structure of PAvarO NTS-DBL1αCIDR1γ (Vigan-Womas et al., 2012) and to the corresponding molecular models of the other DBL domains. Indeed most of those epitopes were conserved and buried on the inside of the protein. As previously suggested, ELISA coating might unveil parts of the molecule that are hidden on the correctly folded full length PfEMP1 therefore giving false positive signals when analysing sero-reactivity by ELISA (Ghumra et al., 2012; Vigan-Womas et al., 2011), a method that has been, and still is commonly used. Further, several co-factors participate in the formation of rosettes and could bind PfEMP1 thus altering its conformation or hiding epitopes in a manner that is not reproducible by ELISA. Caution should be used when taking only ELISA results as a marker of reactivity towards certain proteins. When using surface fluorescence on pRBC surface we detected very low level of cross-

reactivity with few antibodies cross-reacting in between distinct parasite strains. These results could be explained by the necessity of other stimulatory co-factors for cross-reactive antibody generation, that are absent in an artificial immunization setting (other parasite proteins, RBC debris, hemozoin, etc). More probably, repeated exposure to a variety of similar, but not identical antigens in a short time could boost the immune response and be crucial for generation of cross-reactive antibodies, as recently shown in influenza (Wrammert et al., 2011). Our results do not exclude the possibility to generate cross-reactive antibodies, however it is still unclear how and after how many exposures this specific kind of antibodies can be elicited. It could also be that natural immunity is acquired through sequential acquisition of a set of serotype-specific antibodies: in the same pool, antibodies towards a set of epitopes recognize some parasites while a different pool of antibodies recognize other, as suggested elsewhere (Vigan-Womas et al., 2011). Still the question remains as how antibodies against PfEMP1 are acquired in natural infections and how to mimic this acquisition in an artificial setting. Our study indicates that is possible to consistently generate high titres of strain specific antibodies but these have limited cross-reactivity in between different parasite strains.

4.3 PAPER III

A PfEMP1-DBL1α sequence associated with severe malaria is able to generate strain-transcending antibodies.

The *var* gene repertoire is vast and immunity to severe malaria is acquired after few exposures to parasites. The presence of cross-reactive antibodies in between different parasites is possible in a natural infection but difficult to achieve in a laboratory set up (Ghumra et al., 2012; Vigan-Womas et al., 2011 and **Paper II**). It is important to understand the fine details of antibodies that react with multiple parasites to better understand how immunity to malaria is generated in order to design a rational approach to a strain transcending vaccine.

In this study we took advantage of data generated by Normark *et al.* where PfEMP1-DBL1α sequences derived from Ugandan parasites were studied for their association with different disease states utilizing a newly designed algorithm (Normark et al., 2007). We hypothesized that the motifs identified could be available at the parasite surface and therefore synthesized peptides representing six of them (four associated with severe and two with mild malaria). The peptides were used to immunize rats and rabbits. One peptide (RDSM), when used to immunize rats, generated antibodies that, when analysed by flow cytometry, recognized the surface of 50% of the tested parasite strains both patients isolates as well as long term *in vitro* adapted strains. The results indicate that the identified RDSM sequence, which consists of the amino acids ALNRKE, is surface exposed on the PfEMP1 expressed on erythrocyte surface. The purified rat IgG were tested by dot blot on the recombinant NTS-DBL1α proteins expressed by the parasites as well as on a peptide array and the pattern of recognition was identical to the one seen by FACS.
In order to map the residues necessary for the antibody binding, an alanine replacement array was produced. For the rat anti-RDSM, we identified the residues xLNRxx as important for binding to the NTS-DBL1α sequence of the patient isolate UAS22 and

the laboratory strain R29/IT4var9. Further we explored the cross-reactive potential of the antibodies by testing them on 135 degenerated peptide sequences derived from parasites isolated from Ugandan patients (Normark et al., 2007). We found that 47 out of 135 peptides were recognized with different degree of reactivity and recognition was mainly dependent on residues ALxxRKD/E.
Antibodies from rabbits immunized with the RDSM peptide recognized all parasite strains tested by immunofluorescence on air-dried monolayers. However, the rabbit anti-RDSM antibodies did not show any surface reactivity when assayed by FACS. Their reactivity, as determined by alanine scanning, was directed towards the C-terminal residues of the motif (ALNRKE**VW**). The data suggest that the amino acids might be available during transport of PfEMP1 but then hidden on the full PfEMP1 on parasite surface.

Interestingly, a study on VAR2CSA-DBL3χ, involved in placental malaria, identified a peptide in the exact same location as the RDSM hereby studied. When analyzing PfEMP1 sequences derived from parasites infecting pregnant women, the motif EIE**K**D was mainly found in primigravidae, while another motif, GIE**G**E, was found in multigravidae; this associates the motif with severe pregnancy malaria (Dahlbäck et al., 2006), a finding that correlates with the one presented herein.
We were able to reproduce the reactivity in several rats immunized but unexpectedly the reactivity decreased with increasing number of immunizations. Additional studies are needed with modifications of adjuvant, carrier protein and peptide length in order to possibly overcome this problem. The function of the antibodies generated in this study remains elusive and further studies are needed to determine whether they could have an important biological function in, for example, mediating opsonisation of pRBCs by phagocytosis.
This work provides an important framework to the generation of specific strain transcending antibodies in a laboratory setting.

4.4 PAPER IV

Six antibody epitopes targeted during natural P. falciparum infections are associated with plasma capacity to disrupt rosettes.

In this study we wanted to investigate the NTS-DBL1α epitopes recognized by naturally acquired antibodies. We analysed plasma from children living in the malaria endemic area of Buea, Cameroon. Children aged between 6 months and 14 years suffering of malaria were enrolled in the study and divided into two groups: severe malaria or uncomplicated malaria, according to WHO criteria. The plasma samples were tested for their ability to disrupt rosettes and recognize the surface of our model rosetting parasite FCR3S1.2. In addition, they were assayed in ELISA for recognition of the recombinant NTS-DBL1α domain encoded by IT4var60. As expected, plasma from children suffering from severe malaria displayed lower recognition of the pRBC surface, as measured by FACS, and less ability to disrupt rosettes as compared to plasma from uncomplicated cases. The biological data were analysed together with patients' data using principal component analysis (PCA), an unsupervised statistical method that gives an overall trend about the data. PCA demonstrated that the ability to disrupt rosettes was positively correlated with recognition of the recombinant NTS-

DBL1α in ELISA, recognition of the pRBC surface as well as patients age. These parameters were inversely correlated with clinical symptoms such as parasitemia, rosetting rate in patients and body temperature. The data suggest that presence of specific antibodies can protects against severe malaria.

Subsequently, some selected plasma samples were tested on peptide array for peptide recognition. Antibody reactivity towards six of the peptides was positively correlated with the plasma capacity to disrupt rosettes in the parasites. The data were further confirmed by orthogonal partial least square (OPLS) analysis where the ability to disrupt rosettes and peptide recognition were studied. Among the top 30 peptides that were associated with plasma ability to disrupt rosettes, all the six identified above were present.
The six identified peptides were mostly localized in subdomain 1 and 2, in agreement with human antibody epitopes mapped from DBL3χ of VAR2CSA and DBP of *P. vivax* (Chootong et al., 2010; Dahlbäck et al., 2006). A residue (K97) found in one of the peptides hereby described is also possibly involved in heparin binding as discussed in **Paper V**. In addition, one of the peptides (PEP_293) was localized at the end of subdomain 2 and beginning of subdomain 3. On the three-dimensional structure of the domain PEP_293 is adjacent to the RDSM motif, previously described (**Paper IV**). This epitope is partially conserved among distinct DBL1α sequences and cross-recognition of this peptide was consistent and correlated with increased capacity of the plasma sample to disrupt rosettes.

The data presented here confirm the presence of anti-rosetting antibodies in plasma from children with malaria, probably acquired as cross-reactive antibodies towards full length PfEMP1 expressed on the erythrocyte surface after repeated exposures to parasites. The differentially recognized antibody-epitopes mapped to the face of SD1 and SD2 in proximity to the identified receptor-binding site (**Paper V**). In **Paper I** we discuss the SD3-loop as a target for rosette disruptive antibodies, findings that are different from the one presented in this study. This discrepancy has been previously described for DBP and discussed above (Chootong et al., 2010; Siddiqui et al., 2012). The data presented here represent an association between rosette disruption and peptide recognition, therefore high non-immune plasma background (such as the reactivity seen against SD3-peptide) might give false negative results. Further, it is possible that upon subsequent natural infections reactivity to the conserved sequences present in SD1 and SD2 is boosted. On the other hand SD3 could allow the generation of rosette disruptive antibodies that are strain specific and therefore not detected in this work. Overall, this work improved the knowledge on epitopes recognized by naturally acquired antibodies to NTS-DBL1α.

4.5 PAPER V

The receptor binding site of NTS-DBL1α of IT4var60 localizes in a positively charged pocket of subdomain-2 encompassed by residues Y73, K97, K171 and K263.

While the minimum binding sites on the RBC rosetting receptors (BgA, CR1, heparin sulphate) have been studied in detail, remarkably little is known about the precise amino acids involved in the binding on the PfEMP1 molecule. Only for the NTS-

DBL1α of the rosetting parasite PAvarO the fine details of the residues involved in the receptor interaction have been studied in detail. It is indeed of importance to understand whether different parasites bind through different areas of the molecule or if they share common mechanisms of receptor interaction. In this study we analysed the NTS-DBL1α of the rosetting parasite FCR3S1.2 encoded by IT4var60 and, by generating a set of mutated proteins, we unveiled the receptor-binding site utilized by this variant.
Since no crystal structure for this variant is available we generated a molecular model based on the PAvarO NTS-DBL1α domain, and analysed the surface electrostatic potential of the molecule. The known parasite-receptor heparan sulphate is negatively charged we, therefore, focused our mutations onto positively charged patches of the molecule. A set of single or double mutated proteins was generated and their purity and homogeneity assessed by various biochemical methods: size exclusion chromatography shows monomeric form of the proteins, SDS gel confirms their purity and far UV spectrum measured by circular dichroism allowed us to assess the correct secondary structure folding.

The proteins were tested for O RBCs binding using a flow cytometry based method: all constructs except one (Mut A, Y73 and K263) bound to RBC in a dose dependent manner. Interestingly the binding of another construct (Mut F, K97) was decreased by approximately 80%. The results were mirrored by the ability of the recombinant proteins to inhibit the formation of rosettes of the homologous parasite FCR3S1.2. Wild type (WT) protein efficiently blocked rosette reformation, as most of the mutated proteins. However, Mut A was not able to inhibit parasite rosetting.
Since heparan sulphate is the known receptor for FCR3S1.2 rosetting we tested the mutated proteins for their affinity to heparin by using microscale thermophoresis. To our surprise Mut A was binding to heparin with similar affinity while Mut F binding was completely absent. While the heparin binding site identified for the PAvarO NTS-DBL1α is localized in the NTS (Jullerat et al., 2011), the heparin binding site established in this study is localized in SD2 and partially overlaps with the RBC binding site. This discrepancy is not fully surprising since heparan sulphate is not the RBC receptor for PAvarO parasite (Juillerat et al., 2010). By checking the localization of K97 (Mut F) we noticed that this residue is part of a bigger positive patch that comprises also residues Y73 and K263 (Mut A). Our results suggest this region of SD2 to form a high-affinity binding site that possibly engages multiple receptors on the RBC. Binding to multiple receptors is not uncommon and it has been reported also for PAvarO pRBCs that can bind to BgA and an unknown receptor on O RBCs (Vigan-Womas et al., 2012). More interestingly, the RBC binding site identified in this study corresponds to the one identified in PAvarO NTS-DBL1α, despite the differences in receptor specificity (Vigan-Womas et al., 2012). In addition, this cleft is target of human anti-rosetting antibodies (**Paper IV**). The data presented here suggests the presence of a possible common binding site in parasites with rosetting phenotype. This opens possibilities for the design of inhibitors that could block rosetting by targeting the specific area of PfEMP1 involved in binding.

In **Paper I** we reported generation of mAb that could inhibit rosetting and binding to RBC. To investigate their mechanism of we selected mAbs V2-3 and V2-17.1 and tested them for recognition of our mutated proteins. mAbV2-3 had impaired recognition of Mut E (K325 and K327), confirming the epitope identification carried

out by peptide array. Conventional ELISA could not identify the V2-17.1 epitope. We therefore tested for its affinity to the different mutated constructs by microscale thermophoresis: all the mutants behaved identically with no major differences in affinity. Overall these data point out that antibody mediated rosetting inhibition is not due to direct hindrance of the binding site but rather by conformational changes at a distance from the paratope. Alternatively, the binding to the receptor could be a multi-step event involving a first low affinity interaction (mediated by SD3) followed by high affinity receptor binding (mediated by SD2) and mAbs could block this first "engagement" step making it impossible for the pRBCs to rosette.

5 CONCLUDING REMARKS AND FUTURE DIRECTIONS

One of the most important goals for malaria research is to reduce the burden of the disease. The mortality rate for severe malaria is still high, about 15-20%, despite major improvement in the health care system and in therapeutic drugs against the disease. Sequestration of pRBCs in the microvasculature is a relevant phenomenon in the disease pathology, with PfEMP1-mediated rosetting being one of the major contributors. The development of treatments that could diminish sequestration and disrupt rosetting is urgently needed to decrease the disease mortality. Better understanding of PfEMP1 biology is required in order to move toward this goal. What are the targets of antibodies against rosetting parasite variants? Are antibodies able to cross-react in between different variants? Where is the receptor-binding site localized on the molecule? The studies presented in this thesis try to answer some of those questions and findings are summarized in Figure 6, however important issues still need to be addressed.

I. Epitopes targeted by anti-rosetting monoclonal antibodies localize to the subdomain 3 of the NTS-DBL1α molecule. The high immunogenicity of the region suggests the possibility of generating a hybrid construct comprising different protein variants derived from rosetting parasites in order to elicit broadly reactive antibodies. To better understand how these antibodies can disrupt rosettes, structural studies on the NTS-DBL1α in complex with Fab fragments, derived from the generated mAbs, are needed.

II. Immunization with recombinant NTS-DBL1α domains consistently elicits surface reactive antibodies that, however, are mainly strain specific. Cross-reactivity seen when testing by ELISA is mainly due to conserved internal epitopes. In order to overcome strain specificity different immunization strategies could be employed, to better mimic natural infection: mix of different domains, sequential immunization, different adjuvants or animals.

III. Immunization with the DBL1α surface-exposed peptide RDSM generated surface reactive antibodies that are able to cross-recognize a number of parasites, both laboratory strains as well as patient isolates. The same antibodies reacted selectively with the sequence motif spotted on a peptide array, when the residues xLxxKE/D were present. The function of the studied antibodies is still unclear and need to be further investigated. In addition, the presence of anti-RDSM antibodies during natural infections is still unknown.

IV. Naturally acquired antibodies can recognize the surface and disrupt rosettes of heterologous parasites. Presence of such antibodies correlates with protection from clinical symptoms and with the recognition of six specific peptides derived from the NTS-DBL1α sequence. Affinity purification of antibodies on these peptides could clarify their relative contribution to cross-reactivity and rosette-disruption

activity. In order to elucidate the relative importance of the epitopes identified, the same experimental procedure described here could be applied on plasma samples from patients living in different geographical regions. In addition the same plasma samples could be tested on a number of distinct parasite variants.

V. NTS-DBL1α encoded by IT4var60 binds to RBCs and to its receptor heparin/heparan sulphate via a positively charged cleft that comprises aminoacids Y73, K97, K171 and K263. mAbs that inhibit rosettes can exert their action even if they do not bind directly to the receptor binding site. The possible presence of multiple receptors on RBCs still needs to be investigated. Studies on additional NTS-DBL1α variants, for their binding properties, will help to identify a possible conserved binding mechanism. Finally, to understand how mAbs block rosettes a co-crystal structure of the molecule in complex with Fab fragments is needed.

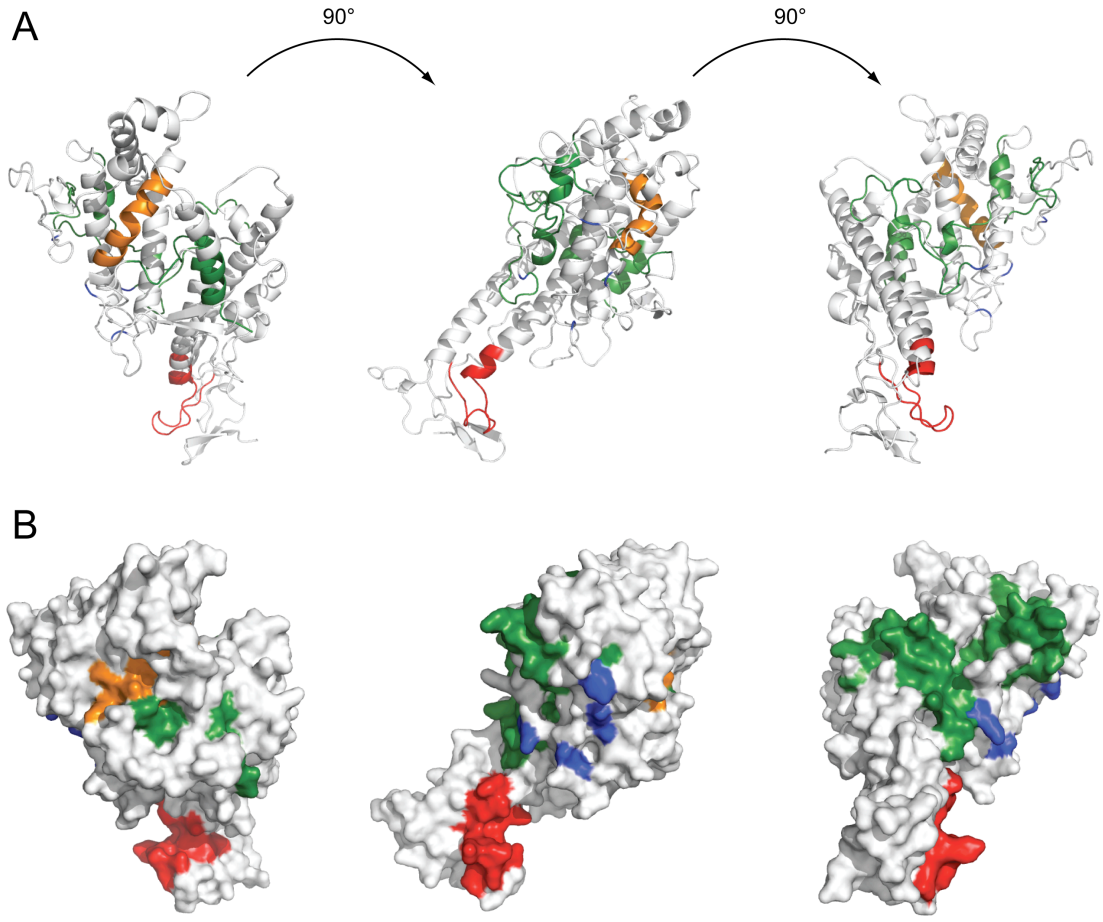

Figure 6. Localization of the functional regions of NTS-DBL1α identified in this thesis. Three 90 degrees orthogonal views of the molecule in cartoon (A) or surface (B) representation with described residues depicted in colour. Red: epitope of rosette disruptive mAbs (**Paper I**); orange: cross-reactive RDSM peptide (**Paper III**); green: epitopes of human Abs associated with rosette disruption capacity (**Paper IV**); blue: RBC and receptor binding residues (**Paper V**)

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