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Surface antigens in *Plasmodium falciparum* malaria: PfEMP1 and SURFIN$_{4.2}$

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Cover illustration: A *Plasmodium falciparum* rosette, with egressing and invading merozoites by Teresa Ducuara, all rights reserved ©

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Surface antigens in *Plasmodium falciparum* malaria: PfEMP1 and SURFIN$_{4.2}$
THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

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In memory of my dad, Marco Aurelio Quintana Blanco

ABSTRACT

Plasmodium falciparum malaria is an infectious disease that on despite of the ongoing eradication efforts is still endemic in more than 100 countries, sometimes causing severe disease that leads to the death of around half a million people per year. Malaria pathology is tightly associated with the parasite cycle inside the human red blood cells (RBCs). Central to this cycle is the initial invasion by the merozoite and the extensive RBC modifications induced by the parasite, transporting proteins to the RBC cytoplasm and membrane. The *P. falciparum* Erythrocyte Membrane Protein 1 (PfEMP1) transported to the surface of the parasitized RBC (pRBC) and the surface-associated interspersed protein 4.2 (SURFIN$_{4.2}$) present both at the pRBC surface as well as at the merozoite apex and surface, are the major focus of this thesis. PfEMP1 is the major surface antigen and mediates rosetting (binding of parasitized RBCs (pRBCs) to two or more RBCs), a parasite phenotype associated with the development of severe disease. The most N-terminal segment of this protein (the NTS-DBL1α domain) has been identified as the ligand for rosetting and naturally acquired antibodies targeting this particular protein protect against severe disease development. In this study we wanted to address the specific regions in PfEMP1 and in other protein targets recognized by rosette-disrupting antibodies (generated upon immunization with recombinant PfEMP1 or naturally acquired during *P. falciparum* infection). We also wanted to explore other functional roles of these antibodies.

A panel of antibodies (monoclonal and polyclonal) against rosette-mediating NTS-DBL1α domains was produced by animal immunization. The antibodies were analyzed with particular attention to their capacity to recognize the surface of the pRBC, disrupt the rosettes formed by homologous parasites and induce phagocytosis by monocytic cells. Additionally, the specific epitopes recognized by the majority of these antibodies were successfully mapped to a specific region of subdomain 3 (SD3) of the DBL1α domain, regardless of the parasite strain used. These results suggested this region as a major target of anti-rosetting antibodies. Most of these antibodies also induced opsonization for phagocytosis, a role that could be of great importance during pRBCs clearance *in vivo*. Interestingly, some of the antibodies with high opsonizing activity did not disrupt rosettes, indicating that other epitopes besides those involved in rosetting are exposed on the pRBC surface and are able to induce functional antibodies that could provide protection.

The naturally acquired antibodies in sera from children living in a malaria endemic region were also investigated. The ability of these antibodies to recognize three parasite-derived surface proteins (PfEMP1, RIFIN-A and SURFIN$_{4.2}$) was assessed. Different variables were also measured in the presence of these sera samples, including rosetting rate, surface reactivity and opsonization for phagocytosis on a rosetting model parasite grown in group O or group A RBCs. The data showed that the acquired immune response developed during natural infection could recognize the pRBC surface and more importantly could induce pRBC phagocytosis and in a few cases disrupt the rosettes formed by a heterologous parasite model. These activities however had limited access to the pRBCs inside a rosette formed with

group A RBCs, where these cells act as a shield for the pRBCs, protecting it from antibodies' recognition therefore impairing their effector function. This study also suggested that SURFIN$_{4.2}$ previously identified at the pRBC surface could be involved in rosette formation, either as a direct ligand or as an accessory element for rosette strengthening.

The suggestion of SURFIN$_{4.2}$ as a possible mediator in rosetting prompted us to deepen the study of this protein, however, the initial results steered the approach to this protein from the rosetting phenomenon towards a more striking and understudied role of this protein during the invasion process. Using antibodies against the N-terminus, the protein was observed at the surface of the merozoite but more strikingly also in the neck of the rhoptries. The protein was shed into culture supernatant upon schizont rupture and was associated with GLURP (Glutamate Rich Protein) and RON-4 (Rhoptry Neck Protein 4) to form a complex we named SURGE (SURFIN4.2-RON-4-GLURP complEx). Importantly, SURFIN$_{4.2}$ was detected at the apex of the merozoite during merozoite initial attachment and active invasion into the RBCs. The exact functional role of SURGE remains to be determined, but the presence of RON-4, a protein confined to the moving junction (MJ), strongly suggests a role in strengthening the stable contact between the merozoite apex and the RBC, possibly as and additional RBC adhesion molecule. Supporting the involvement of the protein complex during the invasion process, antibodies against the N-terminus of SURFIN$_{4.2}$ partially inhibited invasion.

LIST OF SCIENTIFIC PAPERS

This thesis is based on the following papers:

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. **Quintana Mdel P**, Angeletti D, Moll K, Chen Q, Wahlgren M. Phagocytosis-inducing antibodies to *Plasmodium falciparum* upon immunization with a recombinant PfEMP1 NTS-DBL1α
Malaria J. 2016 15(1):416

III. **Quintana Mdel P**, Ch'ng JH, Zandian A, Nilsson P, Saiwaew S, Moll K, Qundos U, Wahlgren M. Antibodies to PfEMP1, RIFIN and SURFIN expressed at the *Plasmodium falciparum* parasitized red blood cell surface in children with malaria.
Manuscript

IV. **Quintana Mdel P**, Chan SC, Ch'ng JH, Zandian A, Imam M, Hultenby K, Nilsson P, Qundos U, Moll K, Wahlgren M.
A novel SURFIN$_{4.2}$ protein complex at the merozoite apex and surface implicated in *Plasmodium falciparum* invasion
Manuscript

The following publications were also obtained during the course of the PhD studies but are not included/discussed in this thesis

I. Blomqvist K, Albrecht L, **Quintana MdelP**, Angeletti D, Joannin N, Chene 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

II. Ch'ng JH, Moll K*, **Quintana Mdel P***, Chan SC*, Masters E*, Liu J, Eriksson AB, Wahlgren M. Rosette-disrupting effect of an anti-plasmodial compound for the potential treatment of *Plasmodium falciparum* malaria complications. Sci Rep. 2016 6:29317

* Equal contribution

CONTENTS

LIST OF ABBREVIATIONS

ACT	Artemisinin combined therapy
ATS	Acidic terminal segment
AMA-1	Apical membrane antigen-1
ASC	Antibody secreting cells
CD36	Cluster of differentiation 36
CIDR	Cysteine rich interdomain region
CLAG	Cytoadherence-linked asexual protein
CR1	Complement receptor 1
CRD	Cysteine rich domain
CSA	Chondroitin sulfate A
CyRPA	Cysteine-rich protective antigen
DBL	Duffy binding like
DC	Domain cassette
EBL	Erythrocyte binding ligand
EGF	Epidermal growth factor
EPCR	Endothelial protein C receptor
ER	Endoplasmic reticulum
EXP	Exported protein
FACS	Flow activated cell sorting
FBS	Fetal bovine serum
GAP	Glideosome-associated protein
GLURP	Glutamate rich protein
GPI	Glycosylphosphatidylinositol
HA	Hyaluronic acid
HS	Heparan sulfate
HSP	Heat shock protein
ICAM-1	Intercellular adhesion molecule-1
IMC	Inner membrane complex
KAHRP	Knob-associated histidine-rich protein
LLIN	Long-lasting insecticide treated nets

MAHRP	Membrane-associated histidine-rich protein
MBC	Memory B cells
MC	Maurer’s clefts
MESA	Mature parasite-erythrocyte surface antigen
MS	Mass spectrometry
MSP	Merozoite surface protein
NTS	N-terminal segment
PAM	Pregnancy associated malaria
PECAM-1	Platelet endothelial cell adhesion molecule 1
PEXEL	*Plasmodium* export element
PfEMP1	*Plasmodium falciparum* erythrocyte membrane protein 1
PHIST	*Plasmodium* helical interspersed subtelomeric protein
PLPs	Perforin-like proteins
PNEPs	PEXEL-negative exported proteins
pRBC	Parasitized red blood cell
PSAC	Plasmodial surface anion channel
PTEX	*Plasmodium* translocon of exported proteins
PV	Parasitophorous vacuole
PVM	Parasitophorous vacuole membrane
PvSTP	*Plasmodium vivax* subtelomeric transmembrane protein
Rh	Reticulocyte binding-like homologues
RBC	Red blood cell
RDT	Rapid diagnostic test
RESA	Ring parasite-infected erythrocyte surface antigen
REX	Ring exported protein
RIFIN	Repetitive interspersed family
RIPR	Rh5 interacting protein
RON	Rhoptry neck protein
SBP-1	Skeleton binding protein-1
SICA	Schizont-infected cell agglutination
SD	Subdomain

SP	Signal peptide
SRP	Signal-recognition particle
STEVOR	Subtelomeric variable open reading frame
SURFIN	Surface-associated interspersed
SURGE	$\text{SURFIN}_{4.2}$-RON-4-GLURP complex
TARE	Telomere associated repeat elements
TVN	Tubovesicular network
TM	Transmembrane
VCAM-1	Vascular cell adhesion molecule 1
WHO	World health organization
WRD	Tryptophan-rich domain

1 INTRODUCTION

1.1 MALARIA

1.1.1 The disease

Malaria is a vector borne disease caused in humans by infection with 5 species of the genus *Plasmodium, P. falciparum*, *P. vivax*, *P. ovale*, *P. malariae and P. knowlesi*, with data suggesting that the two first species cause 95% of the infections (Garcia 2010). *P. falciparum* is the most prevalent species in Africa and is responsible for most of the deaths associated to malaria (World Health Organization (WHO) 2015).

The clinical presentation of *P. falciparum* malaria ranges from complicated/severe disease (potentially causing death) to benign and asymptomatic infections. In general, the symptoms onset occur 8-12 days after infection (related to the period of infection in the liver, see below in parasite life cycle section). Symptoms are typically unspecific and include malaise, aches (headache, muscle ache), fatigue, anorexia and nausea. These initial symptoms are followed by a febrile stage accompanied of chills, more severe headache, nausea and vomiting. Febrile episodes can become periodic, related to the schizont rupture from the red blood cells (RBCs) during a synchronous infection every 48 hours. Other symptoms include splenomegaly and hepatomegaly (Garcia 2010).

Severe/complicated malaria can cause vital organ dysfunction leading to death. This particular disease presentation is defined by clinical or laboratory evidence of vital organ dysfunction associated to parasite asexual parasitaemia with no other confirmed cause for the symptoms. Clinical and laboratory signs include impaired consciousness, prostration, convulsions, deep breathing, respiratory distress, pulmonary edema, circulatory collapse or shock, acute kidney injury, clinical jaundice, abnormal bleeding, hypoglycemia, metabolic acidosis, severe anemia, hemoglobinuria, hyperparasitaemia and renal impairment. Severe malaria can also occur during pregnancy in a clinical presentation known as pregnancy associated malaria (PAM), being an important cause of abortion, stillbirth, premature delivery and fetal death. Any of the signs for severe malaria (mentioned above) can be present in pregnant women, but it seems that hypoglycemia and pulmonary edema are especially common. Fetal distress and death are also very common and if the fetus survives, there is growth retardation. There are also risks for the mother after delivery, including hemorrhage and puerperal sepsis (World Health Organization (WHO) 2014).

1.1.2 Malaria burden

Malaria is endemic in 106 countries, with and estimated of 214 million cases (range: 149-303 million) and 438000 deaths (range: 236000-635000). These figures represent a decline in incidence and death of 37% and 60% respectively compared with the year 2000 (World Health Organization (WHO) 2015). This encouraging improvement, however, has been challenged in a 2012 study indicating that deaths might be underestimated by WHO, in particular among adult patients (Murray et al. 2012). The considerable decline both in incidence and mortality is attributed to the scale up of malaria interventions during recent years, including the introduction of rapid diagnostic tests (RDTs), implementation of artemisinin based combination therapy (ACT) and distribution of long-lasting insecticide treated nets (LLIN) (Alonso & Tanner 2013). A major concern is if the decline can be sustained, considering that in parallel to the scale up in malaria control interventions, both vector and parasite have develop strategies to evade malaria interventions. Parasite resistance to artemisinin has emerged on the Thai-Cambodian border (Noedl et al. 2008; Dondorp et al. 2010) and the *Anopheles* mosquito has also developed resistance to commonly used insecticides (Hemingway 2014).

Malaria is a disease associated with poverty, both sharing geographical frames and being concentrated in the tropical and subtropical regions, with the causality between the two moving in both directions. It is estimated that countries with high malaria transmission have 1.3% lower economic growth than those that are malaria free. The costs associated to malaria include a combination of personal, public and private expenditures used both for prevention and treatment of the disease (Sachs & Malaney 2002; Chuma et al. 2010). The estimated budget to control and eradicate malaria globally reaches figures close to the 5 billions of dollars per year and even though the funding seems to be insufficient (Alonso & Tanner 2013) a recent systematic review indicates that the benefits of investing in malaria, greatly outweigh the costs (Ranson & Lissenden 2016).

1.2 PARASITE LIFE CYCLE

Plasmodium falciparum is transmitted to the human host by a female *Anopheles* mosquito, which injects sporozoites in the bite puncture during a blood meal (Fig. 1a). The sporozoites migrate through the skin and then rapidly into the bloodstream to finally reach the liver where it actively invades the hepatocytes (Fig. 1b and c). Each sporozoite differentiates and multiplies in thousands of merozoites that subsequently egress the hepatocytes (Fig. 1e) and enter the circulation where they can start their cycle inside the RBCs (Fig. 1f). This initiates the infection to which most of the clinical manifestations of the disease are attributed.

Inside the RBC the merozoite develops through ring, trophozoite and schizont stages. Soon after invasion, the parasite flattens into a discoidal, flat or cup-shaped ring form that begins to feed on hemoglobin through the cytostome, gradually maturing into a more rounded or irregular form: the trophozoite. This latter stage is relatively more active having an increased protein synthesis activity, evidenced by an increased number of free ribosomes and endoplasmic reticulum enlargement (Bannister et al. 2000). During this stage most of the surface and cytoplasmic modifications of the host RBC are evident, causing (particularly in severe/complicated malaria cases) the sequestration of the parasitized RBCs (pRBC) in various tissues (in the brain for example, causing cerebral malaria - Fig. 1g). From the trophozoite stage, the parasite progresses into the schizont stage, defined as a parasite that is or has divided through schizogony, generating an even number of merozoites that are released into the bloodstream invading new RBCs and thus initiating a new round of schizogony. After several erythrocytic generations, some of the merozoites begin to develop into the male and female gametocytes (Fig. 1h). These gametocytes are ingested with the mosquito's blood meal, maturing into gametes within the gut. The male gametes break the RBC, become motile, and penetrate the female gametes forming a fertilized stage known as the zygote. Later on, this zygote becomes motile and then is called the ookinete. This stage migrates to the mosquito midgut (Fig. 1i) and grows into the oocyst, which matures producing hundreds of sporozoites. Within the mosquito the oocyst ruptures, releasing the sporozoites that rapidly migrate to the salivary glands where they become infective. When the mosquito takes a blood meal, the sporozoites are injected with saliva into a new host completing the life cycle (Pierce & Miller 2009).

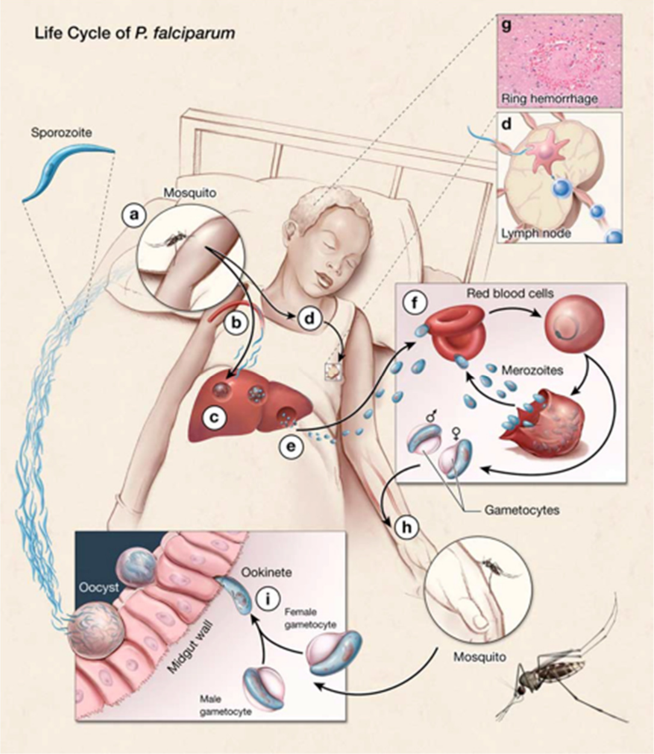

Fig. 1. *Plasmodium falciparum* life cycle. Reproduced from (Pierce & Miller 2009) with permission from the publisher.

1.3 MALARIA PATHOGENESIS

1.3.1 Parasite invasion

The invasion process into the RBCs is a very complex process and can be divided in a successive series of steps initiating with the merozoite egress, followed by the contact with the new host cell, re-orientation and the final entry phase, where the parasitophorous vacuole (PV) is formed (Fig. 2).

1.3.1.1 Merozoite egress

The continuation of the infection inside the human host, requires the continuous re-invasion of new host cells, a process that initiates with the egress of the merozoites from an infected cell. In *P. falciparum* two possible mechanisms for the parasite egress have been proposed:

(1) the egress involves the fusion between the parasitophorous vacuole membrane (PVM) and the RBC membrane in an exocytosis-like process, discharging the merozoites in the extracellular space (Clavijo et al. 1998; Winograd et al. 1999; Winograd et al. 2001) and (2) the egress involves an explosive event where the PVM and RBC membrane are disrupted, scattering the merozoites in the extracellular space (Dvorak et al. 1975; Salmon et al. 2001; Wickham et al. 2003; Glushakova et al. 2005; Glushakova et al. 2007; Glushakova et al. 2013). An increasing amount of evidence supports the second mechanism, and the current research has been focused in the order of the membrane disruption and in the identification of the molecular mechanisms underlying the process. In general, the process appears to include two steps: a swelling and disruption of the PVM prior to that of the RBC membrane. This is a process dependent on cysteine proteases since inhibition with E-64 causes its blockage (Wickham et al. 2003; Glushakova et al. 2010). Recent evidence has shown that besides parasite-originated proteases, the parasite can hijack host cell cysteine proteases (calpain-1) to facilitate the egress process, apparently through proteolysis of the RBC cytoskeleton (α-Spectrin, β-Spectrin and ankyrin) and remodeling of the RBC membrane (Millholland et al. 2011; Chandramohanadas et al. 2009). There is some contradictory evidence suggesting that cysteine proteases are involved in the RBC membrane disruption and not at all in that of the PVM, since the use of E64 generates the accumulation of merozoites surrounded by a single membrane that corresponds to the PVM and is positively stained with a PVM marker (Salmon et al. 2001). The second step in the egress process involves poration of the RBC membrane (Glushakova et al. 2010; Abkarian et al. 2011), that might be caused by perforin-like proteins (PLPs) as has been shown in *Toxoplasma* egress (Kafsack et al. 2009). The poration is followed by an initial rapid discharge of 1-2 merozoites (more than 5 in adherent RBCs). After this an outward curling and a fast eversion of the membrane occurs pushing forward the remaining merozoites (Glushakova et al. 2010; Abkarian et al. 2011).

1.3.1.2 Entry into the RBC

Extensive studies of the RBC invasion by *P. falciparum* merozoites suggest that the process involves a primary low affinity and reversible interaction through any portion of the merozoite. This interaction is believed to be mediated by merozoite surface proteins (MSPs), being MSP-1 the most abundant covering the entire surface (Holder et al. 1985) and apparently being important for the merozoite invasion process since antibodies against this protein inhibit invasion (Siddiqui et al. 1987; de Koning-Ward et al. 2003). Other proteins anchored to the merozoite membrane through glycosylphosphatidylinositol (GPI) in a similar fashion as MSP-1 have been described: some containing EGF (epidermal growth factor) and six-cysteine (6-Cys) domains that might be involved in protein-protein interactions (Sanders et al. 2005) and others presenting structures with indistinguishable domains, the case of MSP-2 (Low et al. 2007). Besides the glycosylphosphatidylinositol (GPI)-anchored proteins, there is other group of proteins associated to the surface through interactions with MSP-1, including MSP-3, 6 and 7 (Kauth et al. 2003; Kauth et al. 2006) forming the MSP-1 complex, however it is not clear the exact role they perform during invasion.

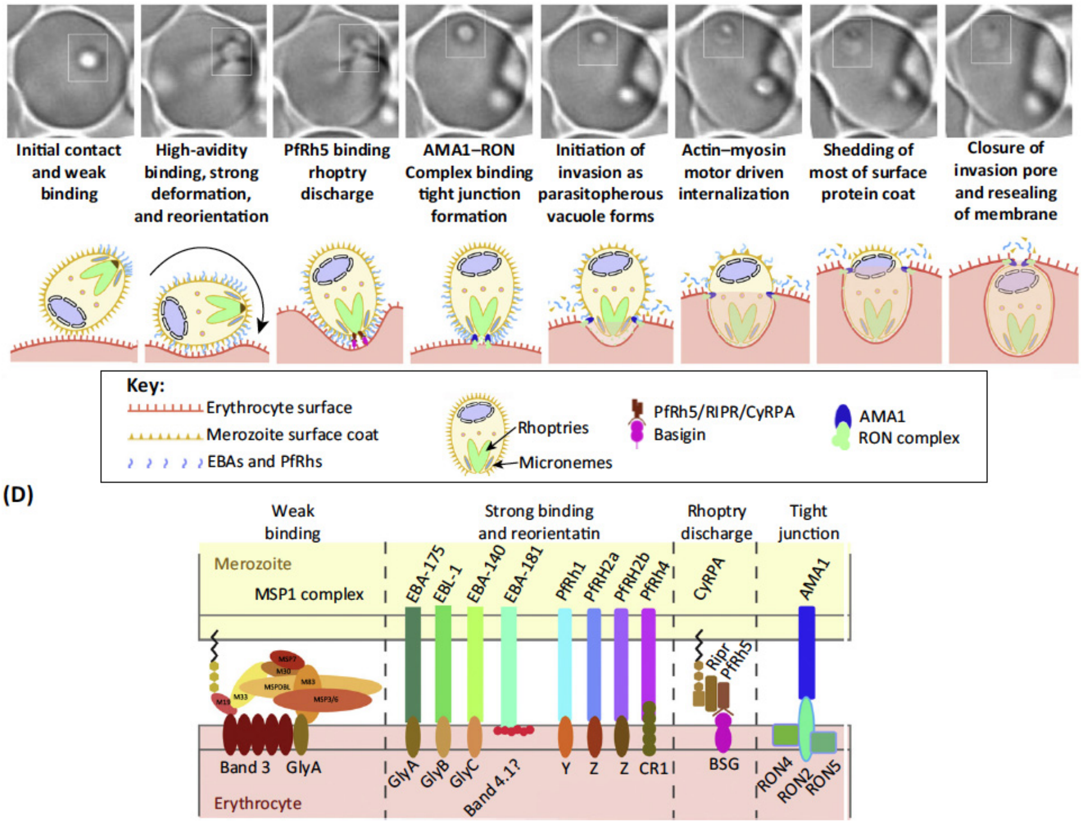

Fig. 2. Merozoite invasion into the red blood cell. Images from each of the invasion stages are shown with cartoons illustrating the relevant parasite ligands-RBC receptor interactions. Reproduced from (Weiss et al. 2016) with permission from the publisher.

Following the initial recognition, the merozoite re-orientates putting its apical end (the place where the apical secretory organelles are located) in direct contact with the RBC surface. This re-orientation involves active movement of the merozoite and deformation of the RBC membrane (Dvorak et al. 1975; Gilson & Crabb 2009; Weiss et al. 2015). After the re-orientation, the apical end attaches strongly to the RBC membrane through interactions of parasite transmembrane (TM) proteins to receptors on the RBC membrane (Fig. 2, lower panel). These proteins are generally divided in two groups, the erythrocyte binding ligands (EBLs) and the reticulocyte binding-like homologues (PfRhs) (reviewed by (Cowman et al. 2012)), binding to different receptors on the RBC membrane and allowing the use of alternative pathways for invasion (Duraisingh et al. 2003). These interactions appear to trigger subsequent events leading to invasion (Singh et al. 2010; Riglar et al. 2011; Srinivasan et al. 2011). Once the parasite has re-oriented, an essential interaction occurs between Rh5 (an unique member of the Rh family) and its receptor on the RBC, basigin (Baum et al. 2009; Crosnier et al. 2011). Rh5 differs from other members in its family, being smaller and lacking a TM domain. The protein however is tethered to the merozoite surface through interactions with the Rh5 interacting protein (PfRIPR) which in turn binds the cysteine-rich protective antigen (CyRPA), the latter being a GPI-anchored protein (Chen et al. 2011; Reddy et al.

2015). After Rh5 interaction occurs, a step of rhoptry discharge occurs, releasing the RON complex (rhoptry neck proteins including RON-2, 4 and 5) that is inserted in the RBC and interacts with AMA-1 in the merozoite surface (Richard et al. 2010; Riglar et al. 2011; Srinivasan et al. 2011; Vulliez-Le Normand et al. 2012) forming the moving junction (MJ) through which the parasite will push its way into the nascent PV. The micronemal apical membrane antigen (AMA-1), is translocated to the merozoite membrane before invasion begins and appears to be essential since it cannot be genetically disrupted (Triglia et al. 2000) and AMA-1 antibodies inhibit invasion (Dutta et al. 2003; Healer et al. 2004). Moreover, experimental evidence indicates that AMA-1 is required for invasion (Yap et al. 2014; Riglar et al. 2015) on despite of a conflicting reports indicating the opposite (in *P. berghei* -a rodent malaria parasite- and *Toxoplasma* which possess AMA-1 –like homologs) (Bargieri et al. 2013; Lamarque et al. 2014). The force that finally drives invasion is produced by a single-headed myosin attached to the inner membrane complex (IMC) via a set of proteins (Baum et al. 2006). One of these proteins is the glideosome-associated proteins 45 (GAP45), located in the space between the merozoite IMC and the plasma membrane. Actin filaments also concentrate at this site, showing a ring-like distribution at the tight junction of the invading merozoite trailing the RON complex (Angrisano et al. 2012). This provides a possible substrate where the myosin head can interact to generate the necessary movement that pushes the merozoite into the space of the forming PV till a final sealing occurs, pinching off the PMV from the RBC membrane.

1.3.2 Protein trafficking

1.3.2.1 Trafficking inside the parasite

In eukaryotic cells, entry into the secretory route is controlled by signal peptides (SP) located in the N-terminus of proteins destined for secretion. These usually hydrophobic sequences are recognized by the signal-recognition particle (SRP) for transport into the endoplasmic reticulum (ER). Once the protein reaches the ER lumen the SP is cleaved and the protein is subsequently transported through coat protein complex (COP-II)-coated vesicles into the Golgi apparatus, where further processing and modifications occur before the final vesicular transport to the membrane and secretion in the extracellular space. The early secretory system of *P. falciparum* seems to be similar to this general mechanism. However, the final destination of the secreted proteins is not the extracellular space; instead is the lumen of the PV, the RBC cytoplasm or the RBC membrane (Lingelbach & Przyborski 2006). Moreover, many proteins secreted from the parasite contain unusual N-terminal SP. Even though hydrophobic, these SPs are generally recessed from the N-terminal end of the protein by between 10 and 50 amino acids (Hiss et al. 2008). Another unusual phenomenon is that there are many exported proteins that lack an N-terminally located SP and therefore must use another type of sequence (probably an internal TM domain) to allow the entry of the protein into the secretory route. Examples of this kind of proteins are PfEMP1 (Spielmann & Gilberger 2010) and SURFIN$_{4.2}$ (Alexandre et al. 2011).

The transit through the ER not only involves cleavage of the SP, but also the interaction with chaperones that allow a proper and correct formation of the protein tertiary structure. This process can be also accompanied by the formation of disulfide bonds and the addition of glycan groups. *Plasmodium* has a limited or totally absent capacity for glycosylation and thus lacks many of the enzymes required for this process (Gowda & Davidson 1999; Davidson & Gowda 2001). As mentioned before, proteins destined for secretion are loaded in vesicles coated with COP-II and homologues for the five core proteins of COP-II have been identified in *Plasmodium* (Albano et al. 1999). Transport between the ER and Golgi in *Plasmodium* appears to be similar as in most of eukaryotic cells, but the Golgi apparatus is rather rudimentary and the traditional morphology consisting of stacked flattened cisternae has not been documented. However, various molecular Golgi markers have been observed including ERD2, GRASP1 and 2 and the GTPase Rab6 (De Castro et al. 1996; Van Wye et al. 1996; Struck et al. 2005; Struck et al. 2008). Moreover, COP-I homologues have been identified that could be involved in retrograde and anterograde transport inside Golgi. Homologues of many of the known trafficking machineries that act in the protein trafficking after Golgi have been identified in *Plasmodium*, including clathrin, clathrin adaptors, SNARE and Rab proteins (Quevillon et al. 2003; Ayong et al. 2007).

1.3.2.2 Trafficking outside the parasite

As mentioned before, most of the proteins secreted by the parasite follow a complex route beyond the parasite membrane, so additionally signal sequences are needed in order to sort the proteins to their final destination in the pRBC. Most exported proteins have a short sequence (RxLxE/D/Q) known as the *Plasmodium* export element or host targeting signal (PEXEL/HT) which directs their transport beyond the VP lumen (Hiller et al. 2004; Marti et al. 2004). However, there is a subset of exported proteins that lacks both the PEXEL/HT motif and the N-terminal SP (e.g. SBP1, MAHRP1, MAHRP2, REX1, and REX2) generally referred as PNEPs (PEXEL-negative exported proteins). The PEXEL motif is cleaved by Plasmepsin V in the ER after the leucine residue, generating a new N-terminus: xE/Q/D (Boddey et al. 2010; Russo et al. 2010). This might be recognized by the *Plasmodium* translocon of exported proteins (PTEX) on the PVM, responsible for the protein translocation across the PVM into the cytoplasm of the pRBC. This translocon is an ATP-powered complex comprised of a heat shock protein (Hsp 101), a known integral membrane protein of the PVM (EXP2), thioredoxin 2 and two novel proteins (PTEX150 and PTEX88), (de Koning-Ward et al. 2009). Hsp101, EXP2 and PTEX150 are stored in dense granules in invading merozoites and are released into the forming PV during invasion to establish association with PVM. Among the three, EXP2 seems to be more strongly associated with the PVM, indicating that it might be the anchor for the rest of the complex and maybe the responsible for the pore formation through which the proteins are translocated. Moreover, homo-oligomers for EXP2 as well as for Hsp101 have been detected (Bullen et al. 2012).

How exported proteins are trafficked within the pRBC cytoplasm is still puzzling. Some evidence suggests that vesicles budding from the PVM could be the connection between the

PVM to MC, and possible to the RBC membrane (Taraschi et al. 2001; Taraschi et al. 2003; Wickham et al. 2001). Others have proposed a model where proteins move by lateral diffusion along a continuous membrane network that includes the MCs and connects the PVM to the RBC membrane (Wickert et al. 2003). However, recent data provides evidence that MCs are individual entities and that they are neither connected to the PVM nor fuse with the RBC membrane, making this model unlikely (Grüring et al. 2011). Soluble proteins are most likely trafficked across the host cell cytosol by diffusion or as part of a soluble protein complex, as has been shown for KAHRP, PfEMP3 and MESA (Howard et al. 1987; Knuepfer et al. 2005). For proteins containing TM and/or hydrophobic regions (e.g. PfEMP1) evidence suggests that the protein passes through the PVM translocon in a soluble state after which it is transported in a multimeric protein complex to the MCs before reaching the RBC PM (Knuepfer et al. 2005; Papakrivos et al. 2005). Recent evidence supports this idea, showing that PfEMP1 associates and co-localizes with a parasite derived complex hsp70/hsp40 (chaperone/co-chaperone) throughout its transport towards the membrane (Külzer et al. 2012), moreover, hsp40 has been detected in association with other markers for knobs (KAHRP and PfEMP3) and components of the PTEX (Hsp101 and PTEX150) (Acharya et al. 2012).

1.3.2.3 Red blood cell remodeling

Plasmodium falciparum invades mature human RBCs, enucleated cells considered metabolically inert, which main function is the transport of O_2 from pulmonary capillaries to tissue capillaries, where it is exchanged for CO_2. These cells have lost the nucleus, the internal organelles and basically all the functional trafficking machinery during their maturation from pluripotent haematopoietic cells (Klinken 2002). Therefore, the parasite must set up and regulate protein transport within the RBC cytoplasm and membrane in order to allow the uptake of nutrients from the host and to establish interactions that might be beneficial for its survival (e.g. cytoadhesion and rosetting). These host remodeling properties are mediated by parasite-derived proteins, which are exported beyond the parasite boundaries through a transport that follows a complex route, crossing the parasite's plasma membrane, the intravacuolar space, the PVM, the host cell cytoplasm and for some proteins final localization in the RBC membrane.

- Modification in the RBC cytoplasm

The principal cytoplasm modification consists of membrane structures (that are believed to be the trafficking machinery inserted by the parasite) including the tubovesicular network (TVN) and the Maurer's clefts (MC). The TVN consists of an interconnected network of tubular and vesicular membranes that spans the cytoplasmic space between the PVM and the RBC membrane during the trophozoite stage (Elmendorf & Haldar 1994; Behari & Haldar 1994). These structures are believed to be involved in import of lipids, amino acids and other molecules (Lauer et al. 1997; Tamez et al. 2008). The MCs are flat and disc-shaped membrane structures resembling morphologically the Golgi cisternae (Lanzer et al. 2006; Haeggström et al. 2007) and is generally accepted that they originate through budding from the PVM or the TVN (Tilley et al. 2008). MCs are mobile during ring stage and rather static during the mature trophozoite stages, being anchored to the host cell cytoskeleton (Wickham et al. 2001). MCs also appear to collapse before merozoite egress, probably facilitating this process (Grüring et al. 2011). There are several membrane proteins resident in MCs, including the skeleton binding protein (SBP1) (Blisnick et al. 2000; Cooke et al. 2006), the membrane-associated histidine-rich protein-1 (MAHRP1) (Spycher et al. 2003), the ring exported protein 2 (REX2) (Spielmann et al. 2006), Pf332 (Hinterberg et al. 1994; Nilsson et al. 2012) and the MC two-transmembrane proteins (MC-2TM) (Sam-Yellowe et al. 2004).

A number of exported proteins, including PfEMP1 (Knuepfer et al. 2005), KAHRP (Rug et al. 2006), PfEMP3 (Waterkeyn et al. 2000), SURFIN (Winter et al. 2005) and RIFIN (Haeggstrom 2004) transiently associate with the clefts before reaching their final location into the RBC membrane, suggesting that MCs might be parasite-induced secretory organelles that concentrate and traffic parasite-derived proteins to the RBC cytoplasm and membrane (Lanzer et al. 2006).

Another important modification in the RBC occurs in the cytoskeleton. Previous studies have clearly demonstrated that the deformability of intact pRBC is profoundly reduced compared to uninfected RBC (Cranston et al. 1984; Nash et al. 1989). Some of this phenomenon can be attributed to the growing parasite, but is mostly due to parasite-derived interactions with the host cell cytoskeleton. Many of the exported and cytoskeleton-interacting proteins are large and generally contain extensive regions of low complexity sequence, often occurring in tandem repeats and typically highly charged (Cooke et al. 2001).

Soon after invasion, the parasite exports cytoskeleton-interacting proteins. The ring parasite–infected erythrocyte surface antigen (Pf155/RESA) is one of the first proteins detectable in the host cell cytoplasm interacting with spectrin (Culvenor et al. 1991; Foley et al. 1991; Ruangjirachuporn et al. 1991). Biochemical studies using recombinant RESA fragments have demonstrated that the interaction with spectrin leads to a degree of protection against heat-induced denaturation of spectrin. This would imply that RESA protects the RBC cytoskeleton from heat-induced damage during febrile episodes (Da Silva et al. 1994). Mature parasite-erythrocyte surface antigen (MESA/PfEMP2) has been reported to compete with host protein p55 for binding to protein 4.1 in trophozoite-stage pRBC resulting in a more rigid host cell (Bennett et al. 1997; Waller et al. 2003). Evidence suggests that KAHRP binds spectrin, actin and ankyrin (Magowan et al. 2000; Pei et al. 2005; Kilejian et al. 1991), as well as the negatively charged acidic terminal segment (ATS) of PfEMP1 (Waller et al. 1999; Ganguly et al. 2015). Discordant evidence has questioned the existence of the ATS-KAHRP interaction (Mayer et al. 2012) and a new anchoring process has been suggested, where members of the *Plasmodium* helical interspersed subtelomeric protein (PHIST) family serve as a bridge between the ATS and the RBC cytoskeleton (Oberli et al. 2014; Oberli et al. 2016). Moreover, in a recent review, the ATS is depicted directly binding the actin filaments (Warncke et al. 2016) but the evidence supporting this interaction is not yet available. *P. falciparum* protein 332 (Pf332) is a large parasite protein that also associates with the cytoskeleton through interaction with actin (Waller et al. 2010) and disruption of the gene makes the pRBC more rigid (compared with cells infected with wild type parasite), indicating that contrary to the majority of cytoskeleton-interacting proteins, this cytoskeleton-interacting protein makes the pRBC less rigid (Maier et al. 2008).

- Modification on the RBC surface

Permeation pathways

After several hours of invasion (approximately 12-18 hours post infection), RBC permeability to low-molecular-weight solutes increases, phenomenon attributed to the induction of channels in the RBC membrane, which allow the uptake of nutrients (e.g. isoleucine (Martin and Kirk, 2007)) and excretion of metabolic waste products. An increase in the RBC membrane conductance due to the activation of a single anionic channel (Plasmodial surface anion channel, PSAC) has been observed. Moreover this conductance was observed specifically in pRBCs (Desai et al. 2000), but conflicting results showing multiple channels and similar activity at lower frequencies on uninfected RBCs, suggested that the factor(s) generating the conductance increase are not exported by the parasite but rather are proteins already present in the host cell that are induced or altered by the parasite (Verloo et al. 2004; Duranton et al. 2005; Ginsburg & Stein 2005; Bouyer et al. 2006). Further attempts to clarify these discrepancies have used high-throughput inhibitors screenings, showing that the tested compounds produced identical inhibitory effects on uptake of sorbitol, amino acids and organic cations and more importantly, they also blocked the single channel conductance observed previously (Pillai et al. 2010). Cytoadherence-linked asexual protein 3 (CLAG3), a parasite protein thought to be involved in cytoadherence, has been implicated in the formation of the anionic channel responsible for the increase in conductance in pRBC (Nguitragool et al. 2011; Pillai et al. 2012). More recently a putative transmembrane domain for CLAG3 has been described and a single mutation located in this region caused alterations in the PSAC activity, generating leupeptin (an antimalarial toxin that requires PSAC uptake) resistant parasites (Sharma et al. 2015). Several questions remain to be explored, particularly if CLAG3 forms the channel alone (by homo-oligomerization), if it interacts with other proteins to form the channel or if it regulates the channel activity.

The surface of the infected RBC

Electron-dense protrusions appear on the surface of the pRBC during trophozoite and schizont development. These structures, known as knobs (Fig. 13), serve as attachment points for sequestration of pRBCs in the blood vessels (Aikawa 1988; Crabb et al. 1997). The knob-associated histidine-rich protein (KAHRP) is the most important member of the knob, being located on the cytoplasmic side (Leech et al. 1984) stabilizing the RBC membrane by interactions with the cytoskeleton (previously mentioned).

1.3.3 *Plasmodium falciparum* surface antigens

1.3.3.1 var/PfEMP1

Plasmodium falciparum erythrocyte membrane protein 1 (PfEMP1) is the most important surface-adhesin responsible for rosetting and sequestration of pRBCs in the microvasculature, and its surface expression coincides with the removal of pRBCs containing mature parasites from the peripheral circulation. Early on it became evident that PfEMP1 is a target of protective antibodies and that acquired immunity develops in response to extended infections with pRBC expressing different PfEMP1 variants (Marsh et al. 1989; Bull et al. 1998; Ofori et al. 2002; Kinyanjui et al. 2003; Chan et al. 2012).

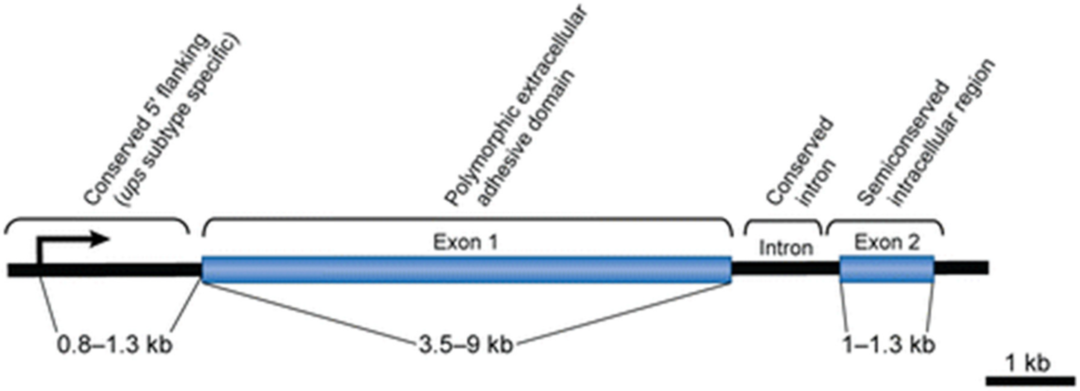

Fig. 3. The common configuration of *var* genes. Reproduced from (Scherf et al. 2008) with permission from the publisher.

PfEMP1s are large (ranging between 200 and 350 kDa) multi-domain proteins, encoded by the hypervariable var gene family that undergoes antigenic variation and thereby allows for the generation of various adhesive phenotypes (Su et al. 1995; Baruch et al. 1995; Smith et al. 1995). The *P. falciparum* genome contains approximately 60 *var* genes mainly located in the subtelomeric region but also in the central parts of the 14 chromosomes (Su et al. 1995; Rubio et al. 1996; Hernandez-Rivas et al. 1997). *var* genes are between 6-14 kb and have a two-exon structure that is separated by a conserved intron (Fig. 3). The first exon encodes a hypervariable extracellular binding region, which comprise the N-terminal segment (NTS), multiple adhesive domains of the duffy binding like (DBL)-type or cysteine-rich interdomain region (CIDR)-type, sometimes interspersed with C2 interdomains. The second exon encodes a C-terminal TM segment and a more conserved ATS. The DBL and CIDR domains are numbered consecutively from the N-terminus and have been classified in six different types (α, β, γ, δ, ε and ζ) and five CIDR types (α, β, δ, γ and pam) based on sequence similarities (Smith et al. 2000; Rask et al. 2010). Among the different domains, the DBL1α is the most conserved (Flick & Chen 2004), and it has been shown that this domain is responsible for rosetting and endothelial binding via heparan sulfate (HS), blood group A antigen and complement receptor 1 (CR1).

The majority of the *var* genes are located near to the telomere associated repeat elements (TARE 1-6), however some *var* genes are located internally in the chromosomes (Fig. 4). The chromosomal location and transcriptional orientation of *var* genes have been shown to correspond to similarities in the 5′ upstream open reading frame of the genes. Based on this conservation, the 5′ promoter regions can be defined into four major upstream (Ups) sequence groups, UpsA, UpsB, UpsC, and UpsE (Lavstsen et al. 2003). Interestingly, rosetting parasites more frequently express *var* genes belonging to group A, although both groups A and B are more often transcribed in patients suffering from severe malaria (Jensen et al. 2004; Normark et al. 2007). UpsA var genes are always located subtelomerically and are transcribed towards the telomere, UpsC are always located internally and UpsB can be located in both places (Fig. 4)

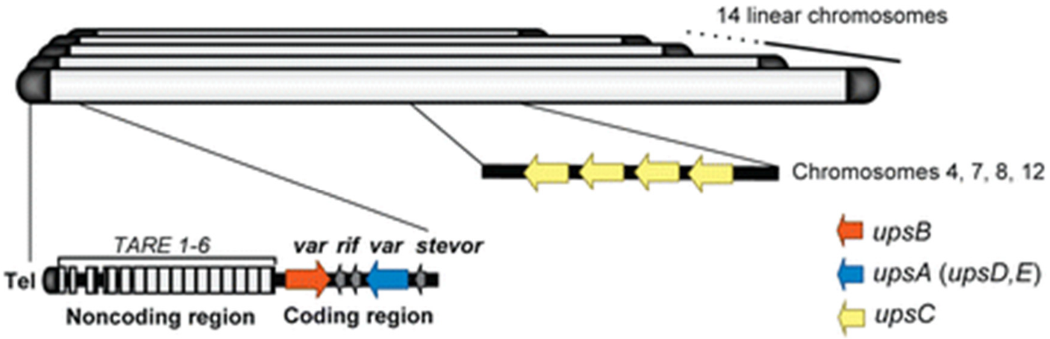

Fig. 4. Organization of *var* genes in the *P. falciparum* genome. Reproduced from (Scherf et al. 2008) with permission from the publisher.

1.3.3.2 surf/SURFIN

The SURFINs, encoded by a small family of surface-associated interspersed (*surf*) genes, were identified by mass spectrometric (MS) analysis of peptides cleaved off the surface of live pRBC with trypsin. The peptides obtained matched with the predicted product of the gene PFD1160w, which consists of two exons and a small intron, encoding a product with a N-terminal segment (predicted to be extracellular) followed by a putative TM and then a long C-terminal segment (predicted to be intracellular) (Fig. 5). The gene PFD1160w resulted to be a member of a family of ten *surf* genes, located within or close to the subtelomeres of five of the 14 chromosomes (chromosomes 1, 4, 8, 13 and 14) (Winter et al. 2005).

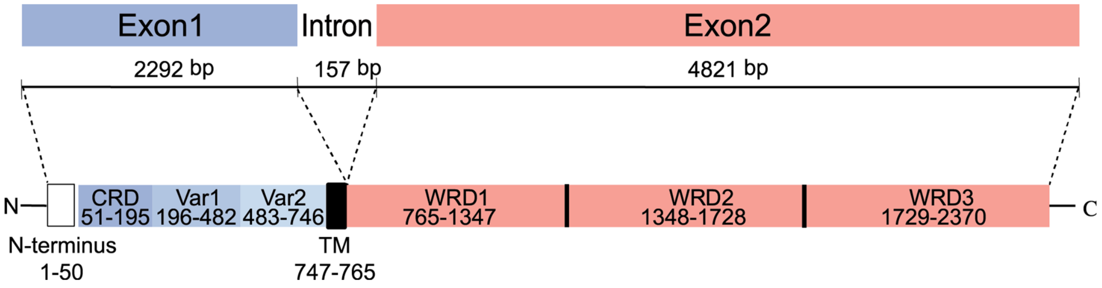

Fig. 5. Structure of the SURFIN$_{4.2}$ gene (PfIT_0422600) and the encoded protein.

This family of proteins is closely related to the *P. vivax* subtelomeric transmembrane protein 1 (PvSTP1) and both share a moderately conserved CRD (cysteine rich domain) with *P. vivax* VIR proteins. The intracellular domain has tryptophan-rich domains (WRDs) that are related with the WRD of PvSTP1, Pf332, the schizont-infected cell agglutination protein from *P. knowlesi* (SICAvar) and the ATS of PfEMP1 (Winter et al. 2005; Frech & Chen 2013) (Fig. 6).

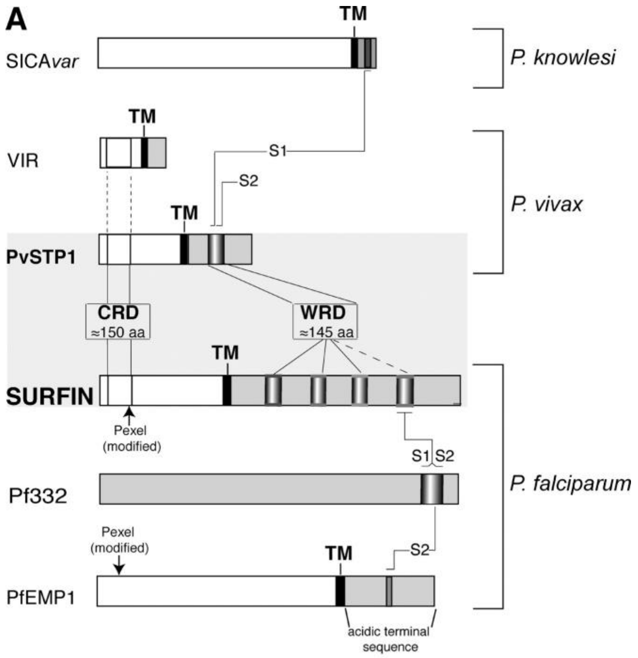

Fig. 6. Common structure of SURFIN proteins and their relation with other *Plasmodiae* surface proteins. Reproduced from (Winter et al. 2005) with permission from the publisher.

Interestingly, comparison of nucleotide sequences encoding the predicted extracellular segment of $SURFIN_{4.2}$ obtained from *P. falciparum* clinical isolates, showed high nucleotide diversity accumulated towards the C-terminal end of the region (just before the intron). Moreover, when evaluating signs of positive diversifying selection, a significant excess of non-synonymous substitutions over synonymous substitutions was detected, suggesting that positive selection is acting on the extracellular region of $SURFIN_{4.2}$, caused maybe by exposure to host immune pressure (Kaewthamasorn et al. 2012). Another similar study conducted with *P. falciparum* isolates from Kenya, led to similar conclusions, postulating $SURFIN_{4.2}$ as a candidate target of naturally acquired immunity (Ochola et al. 2010).

$SURFIN_{4.2}$ is co-transported with PfEMP1 and RIFIN to the pRBC cytoplasm and to the RBC membrane, being associated with the MCs and the knobs. Moreover, it has been observed with the merozoites as a cap-liked zone in the apex of released merozoites (Winter et al. 2005). However, is not clear if it is merely associated to the merozoite from outside or if it is in a particular location in the apical end or the membrane of the merozoite. The association of $SURFIN_{4.2}$ with the apical end of the merozoite and the fact that antibodies towards the protein inhibited invasion, suggested a possible role during this process, however, the high concentrations used (2-5 mg IgG) cast some doubt on the finding, requiring further studies to prove this role.

In a large-scale gene knockout (KO) study (Maier et al. 2008), PFD1160w (SURFIN$_{4.2}$) was disrupted in CS2 parasites. The KO pRBCs showed a significant reduction in the RBC rigidity compared with cells infected with the wild type. This change in the pRBC rigidity is particularly interesting, because it might suggest a possible interaction of the protein with the RBC cytoskeleton, that if indeed occurs, would be mediated by the predicted intracellular domain. As mentioned before this region is related with the giant antigen Pf332 and with the PfEMP1 ATS, both interacting directly or indirectly with the RBC cytoskeleton: Pf332 through direct binding to actin and the ATS through binding to KAHRP and members of the PHIST family, both binding cytoskeleton elements.

*1.3.3.3 rif/*RIFIN *and stevor/*STEVOR

The repetitive interspersed (RIFIN) protein family is structurally related to the subtelomeric variable open reading frame (STEVOR) family. Both *stevor* and *rifin* genes have subtelomeric location and share a very similar two-exon gene structure, where the first short exon encodes a SP or anchor and the larger second exon encodes the rest of the protein (27 to 35KDa). RIFINs were initially predicted to have two TMs flanking a hypervariable region. The first TM existence however was questionable (Joannin et al. 2011) and a recent study has confirmed that there is only one TM domain corresponding to the second one originally predicted (Goel et al. 2015). Evidence has shown that RIFINs are trafficked through the MCs (Haeggstrom 2004; Petter et al. 2007) and that they are expressed on the surface of the pRBC (Fernandez et al. 1999; Kyes et al. 1999). RIFIN proteins have been phylogenetically subdivided in two groups: RIFIN-A and RIFIN-B (with two sub-groups B1 and B2), more importantly, this subdivision seems to be functionally relevant (Joannin et al. 2008; Joannin et al. 2011), since RIFIN-A proteins appear to be exposed on the pRBC while RIFIN-B proteins stay confined in the parasite (Petter et al. 2007). STEVORs were initially found in the MCs and also on the surface of the pRBC particularly in RBCs infected with parasites recently obtained from patients compared with long term adapted parasites (Blythe et al. 2008). In addition, STEVORs are also located in the merozoite apical end where they might be involved in the invasion process (Khattab et al. 2008; Khattab & Meri 2011). Recently both protein families, RIFINs and STEVORs have been implicated in the rosetting and cytoadhesion phenomena (Niang et al. 2014; Goel et al. 2015).

1.3.4 Sequestration: Cytoadherence and Rosetting

The pRBCs being less deformable than normal RBCs and possibly sensitized with antibodies against parasite-derived antigens are more likely to be eliminated from the circulation passing through the spleen (Ho et al. 1990). In order to avoid this clearance, the pRBCs are sequestered in different body locations, either through binding to endothelial cells lining the blood vessels in different organs (cytoadhesion) or by binding to normal RBCs (rosetting), phenomena that coincide with the expression of the PfEMP1 on the surface. The sequestration leads to tissue hypoxia, metabolic disturbances and organ dysfunction, characteristic of severe malaria.

1.3.4.1 Cytoadhesion

Cytoadhesion was observed very early on (19th century) during post-mortem studies made by Marchiafava and Bignami, where the ability of pRBCs to bind to the endothelial cells in different organs was evident. Since that time, a lot of effort has been made to identify the receptor and ligands responsible of this interaction.

The Cluster of differentiation 36 (CD36) has been identified as one of the receptors for cytoadhesion. CD36 is a membrane protein expressed on platelets, macrophages, monocytes, dendritic cells, adipocytes, hepatocytes, myocytes and microvascular endothelium (Silverstein & Febbraio 2009). The majority of *P. falciparum* laboratory adapted strains and clinical isolates from patients bind CD36 (Turner et al. 1994; Newbold et al. 1997; Rogerson et al. 1999) and the parasite ligand appears to be PfEMP1 (Baruch et al. 1996; Baruch et al. 1997; Miller et al. 2002; Robinson et al. 2003). The role of CD36 in malaria pathogenesis is controversial since no correlation between CD36 binding and disease severity has been found in studies conducted in Africa (Newbold et al. 1997; Rogerson et al. 1999). Furthermore there are conflicting reports indicating that CD36 polymorphisms are associated both with protection (Pain et al. 2001; Omi et al. 2003) and susceptibility against severe disease (Aitman et al. 2000; Sinha et al. 2008). In a more recent study, no association between CD36 variants and susceptibility to malaria was observed, and the presence of mutations on CD36 was attributed to evolutionary pressure exerted by other severe infections present in the Sub-Saharan region (Fry et al. 2009).

Another receptor for cytoadhesion is the intercellular adhesion molecule 1 (ICAM-1) that is upregulated by pRBCs adhesion (Berendt et al. 1989). This receptor is expressed on endothelial cells and leucocytes, and the ligand appears to be again PfEMP1 proteins (Springer et al. 2004). A particular subset of group A PfEMP1s, characterized for the presence of a particular arrangement of DBL and CIDR subtypes, denominated domain cassette 4 (DC4) seems to exclusively mediate ICAM-1 binding (Bengtsson et al. 2013). There are indications that sequestration mediated by interactions with ICAM-1 is relevant for *in vivo* pathology, since fatal malaria is associated with upregulation of ICAM-1 and there is co-localization of ICAM-1 and pRBCs on brain endothelial cells from patients who died due to malaria (Turner et al. 1994). Furthermore ICAM-1 adhesion is higher in parasites causing cerebral malaria (Newbold et al. 1997; Ochola et al. 2011).

Endothelial protein C receptor (EPCR) has also been described as an important receptor for cytoadhesion (Turner et al. 2013). EPCR is a transmembrane protein expressed on the large blood vessels and on the microvascular endothelium. Expression has also been detected in other cell types including monocytes, neutrophils, smooth muscle cells, keratinocytes, placental trophoblasts, cardiomyocytes and neurons. EPCR is important for the activity of the protein C (PC) and activated protein C (APC) system, that results in anticoagulant and cytoprotective effects that include anti-apoptotic and anti-inflammatory activities protecting the vascular barrier (Rao et al. 2014). Again the parasite ligand involved in the binding to EPCR is PfEMP1, in particular a subset defined by the presence of DC8 and DC13. Both cassettes contain CIDRα1 domains that mimic features of the natural EPCR ligand, blocking the original interaction (Turner et al. 2013; Lau et al. 2015). Importantly, DC8 and DC13 PfEMP1a are linked to the development of severe malaria (Lavstsen et al. 2012).

Another important receptor is heparan sulfate (HS) present on all cells in the body including RBCs (VOGT et al. 2004). It has been shown that HS as well as heparin bind directly to PfEMP1. Both laboratory adapted strains and clinical isolates bind to HS expressed on endothelial cells (Vogt et al. 2003). Moreover, binding to HS and heparin seems to be more pronounced in parasites isolated from patients with severe malaria compared with those isolated from patients with uncomplicated malaria (Heddini et al. 2001).

Chondroitin sulfate A (CSA) is a sugar that is not normally expressed on human cells, however, is heavily expressed in the placenta syncytiotrophoblasts. pRBCs have been observed sequestered in the placenta causing the clinical presentation known as PAM (McGregor et al. 1983). PAM is associated with the expression of VAR2CSA, a unique PfEMP1 that do not adhere to the previously mentioned endothelial receptors and instead binds CSA (Fried & Duffy 1996; Salanti et al. 2003; Salanti et al. 2004).

Other receptors have been suggested to bind pRBCs, including hyaluronic acid (HA) (Beeson et al. 2000), P-selectin, E-selectin, vascular cell adhesion protein 1 (VCAM-1) (Udomsangpetch et al. 1997) , thrombospondin (Roberts et al. 1985; Rock et al. 1988) and platelet endothelial cell adhesion molecule 1 (PECAM-1) (Baruch et al. 1995; Joergensen et al. 2010; Berger et al. 2013)

1.3.4.2 Rosetting

Rosetting is a parasite phenotype defined by the binding of pRBCs to two or more normal RBCs (Fig. 7). This phenomenon is observed during the trophozoite stage when the PfEMP1 reaches the surface of the RBC. Rosetting was observed for the first time in laboratory strains but later on it was also detected in clinical isolates freshly obtained from malaria patients (Carlson et al. 1990; Wahlgren et al. 1992). Many studies have shown a correlation between rosetting and the severity of malaria in Africa (Carlson et al. 1990; Treutiger et al. 1992; Rowe et al. 1995; Roberts et al. 2000; Normark et al. 2007). The PfEMP1 DBL1α domain has been shown to be the rosetting ligand (Rowe et al. 1997; Chen et al. 1998). Receptors involved in rosetting include: CR1, blood group antigen A and HS.

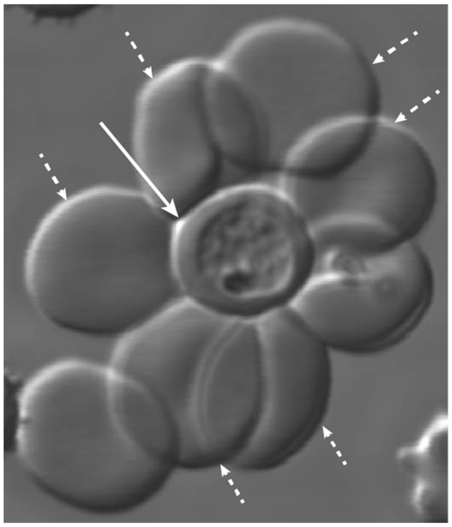

Fig. 7. A *P. falciparum* rosette, with a central pRBC (solid arrow) surrounded by normal RBCs (dash arrows). Picture courtesy of Kirsten Moll.

CR1 (also known as CD35) is present on RBCs and leukocytes and it has been shown that CR1-deficient RBCs do not form rosettes (Rowe et al. 1997). Furthermore, a study conducted in Papua New Guinea showed that CR1 deficiency is common in high-transmission areas and protects against severe disease (Cockburn et al. 2004). Studies using soluble CR1 and antibodies targeting CR1 showed they are able to disrupt rosettes (Rowe et al. 2000).

Blood groups are glycans attached to surface glycoproteins on the RBC surface and have been associated with rosette formation. pRBCs have a preference to form larger and stronger rosettes with A or B blood RBCs (Carlson & Wahlgren 1992; Barragan et al. 2000). A recent study has shown that recombinant NTS-DBL1α binds to blood group glycans and reproduces the preference observed in the parasites (Vigan-Womas et al. 2012). A study from Mali showed that blood group O is a protective factor against severe disease due to a reduction in rosetting (Rowe et al. 2007), and other studies have supported this idea, showing again a correlation between blood groups and malaria severity (Loscertales et al. 2007).

HS seems to be another receptor for rosetting. Studies have shown that HS, heparin and modified heparin (without anticoagulant activity) can disrupt rosettes (Chen et al. 1998; VOGT et al. 2004; Leitgeb et al. 2011).

1.4 IMMUNITY TO MALARIA

Exposure to *P. falciparum* does not induce sterilizing immunity, however, individuals in endemic areas, slowly develop immunity against clinical disease, characterized by lower prevalence of infection and lower rates of disease. This protection against clinical manifestations is age/exposure dependent, with children under the age of five being more susceptible to infection and to complicated/severe disease development. Older children that survive early infections quickly develop immunity against severe disease and thereafter suffer only from uncomplicated/mild disease to finally reach immunity against clinical disease, presenting only asymptomatic infections during adulthood (Deloron & Chougnet 1992; Langhorne et al. 2008) (Fig. 8).

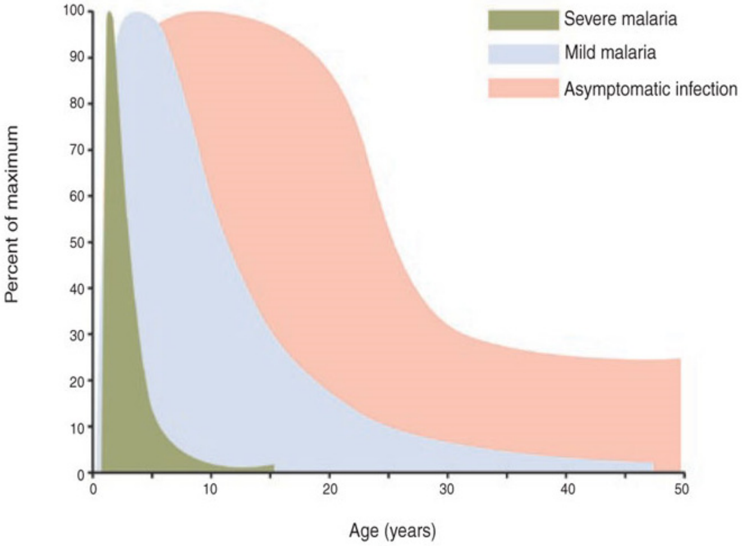

Fig. 8. Acquired immunity against different malaria clinical manifestations in an endemic area. Reproduced from (Langhorne et al. 2008) and reproduced with permission from the publisher.

Early experiments involving passive IgG transfer from immune adults to children suffering from severe malaria with high parasite density, decreased the parasitaemia and alleviated the clinical symptoms, suggesting that acquired immunity is mostly dependent on antibody responses (Cohen et al. 1961). Antibodies mediate protection performing different functional activities including: reduction of sporozoite traversal in the dermis, reduction of sporozoite hepatocyte invasion, inhibition of merozoite invasion, induction of parasite growth arrest, opsonizing (sporozoites, merozoites and pRBCs) for phagocytosis, promoting neutrophil respiratory burst, blocking cytoadhesion and rosetting and preventing schizont rupture (Reviewed in (Teo et al. 2016)). Several studies indicate however that antibody acquisiton is slow and inefficiently generated, waning rapidly when parasite exposure ceases (Reviewed in (Langhorne et al. 2008; Portugal et al. 2013). This phenomenon is attributed to a defect in generating and maintaining memory B cells (MBC) (Dorfman et al. 2005; Asito et al. 2008). Studies have shown that people chronically exposed to malaria accumulate atypical MBCs in a progressive fashion (Weiss et al. 2009; Weiss et al. 2011). Atypical MBCs are characterized by the lack of expression of the classical MBC marker CD27. More importantly these cells seem to express an array of inhibitory genes (FcRL3, FcRL5, CD72, CD200R1, LILRB1, LILRB2 and FCGR2B) that ultimately impair the MBC regular function, losing their ability to signal via the B cell receptor and to differentiate into antibody secreting cells (ASC) (Portugal et al. 2015). A conflicting report, however, indicated that atypical MBCs could indeed secrete antibodies that efficiently inhibited *P. falciparum* invasion (Muellenbeck et al. 2013).

2 SCOPE OF THE THESIS

Increase the understanding of malaria pathogenesis through the study of two surface antigens: The PfEMP1 (focusing on the NTS-DBL1-α domain) transported to the surface of the pRBC and the SURFIN$_{4.2}$ present both at the pRBC surface as well as at the merozoite apex and surface.

Specific aims

- Explore the epitopes targeted by rosette disruptive antibodies upon immunization with a PfEMP1 NTS-DBL1-α domain (Paper I)

- Explore the functional roles of antibodies generated against the NTS-DBL1-α domain (Paper I and II)

- Explore the functional mechanisms (anti-rosetting and antibody dependent phagocytosis activity) of natural acquired antibodies against *P. falciparum* surface antigens (PfEMP1, SURFIN$_{4.2}$ and RIFIN-A) in children with malaria (Paper III)

- Explore the precise subcellular localization of SURFIN$_{4.2}$, with a focus on late stages pRBCs and merozoites (Paper IV)

- Explore SURFIN$_{4.2}$ functional role, focusing on the previously suggested role in invasion (Paper IV)

3 EXPERIMENTAL PROCEDURES

All materials and methods used for the experiments presented in this thesis are described in more detailed in Papers I-IV. Below there is a general description of the methodologies used in all four papers presented.

Parasite cultures

P. falciparum FCR3S1.2 strain was used throughout the experiments, otherwise indicated. Parasites were cultured according to standard methods using group O (for continuous culture) or group A RBCs (for particular experiments in Paper III) in the presence of A^+ non-immune (Swedish) human serum. Culture flasks were gassed with 90% NO_2, 5% O_2 and 5% CO_2 and placed in a 37°C shaker incubator. Parasites were routinely synchronized at ring stage by sorbitol treatment (Lambros & Vanderberg 1979) and the FCR3S1.2 rosetting phenotype was maintained by enrichment over a Ficoll-cushion (Udomsangpetch et al. 1989).

Recombinant protein expression

For the experiments presented in this thesis three *P. falciparum* recombinant proteins expressed in *Escherichia coli* were produced. In all cases the endogenous parasite sequences were commercially codon optimized for optimal expression in bacteria (DNA2.0).

The expression of the NTS-DBL1α-domain used in papers I-III was performed as previously described (Angeletti et al. 2013). In brief the NTS-DBL1α-domain of a rosette mediating PfEMP1 (ITvar60, PFIT_bin06900) was cloned into the pJ414express vector (DNA2.0) and protein expressed with a C-terminal 6x histidine-tag from the soluble fraction.

The expression of the $SURFIN_{4.2}$ (PFIT_0422600) used in papers III-IV was performed as follows. The coding sequence for the N-terminus (predicted extracellular domain) of $SURFIN_{4.2}$ was cloned into the pDest527 vector (kind gift from Dominic Esposito, Addgene plasmid #11518) and the protein was expressed with an N-terminal 6x hisitidine-tag. Protein was retrieved from washed inclusion bodies (IBs) with denaturing solution for 2 hours at room temperature.

The expression of the RIFIN-A (PF3D7_0100400) used in paper III was performed as follows. The sequence was cloned into the pJ414express vector (DNA2.0). Protein was expressed with a C-terminal 6x histidine-tag. In a similar way as with the $SURFIN_{4.2}$ recombinant protein was solubilized from washed IBs with denaturing solution.

Both proteins (RIFIN-A and $SURFIN_{4.2}$) were thereafter refolded by the rapid dilution method. 25 mg of protein were reduced with DTT for 1 hour at room temperature and the solution was added drop wise into ice-cold refolding buffer. After refolding for ≈24 hours at 4°C the proteins were dialyzed against PBS and concentrated using centrifugal filter units.

The three proteins were purified by IMAC (Immobilized Metal Affinity Chromatography) over a Cobalt or a Nickel column. The purified proteins were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and western blot using an antibody against the poly-His tag.

Generation of Monoclonal and Polyclonal antibodies

Antibodies against the recombinant NTS-DBL1α domain and SURFIN$_{4.2}$ were produced as described previously (Angeletti et al. 2013). Monoclonal antibodies against the NTS-DBL1α domains (ITvar60 and others presented in Paper I) were produced in collaboration with the EMBL Monoclonal Antibody Core Facility, Monterotondo, Italy. Mice were immunized three times with 50 μg of recombinant protein. Antibody levels were measured prior and a post fusion by ELISA to select positive cell clones. Monoclonal antibodies were purified over Protein G agarose columns (Pierce Thermo Scientific) and subsequently dialyzed and concentrated.

Goat, rabbit and rat polyclonal antibodies were produced by Agrisera (Vännäs, Sweden). Animals were immunized four times at one-month intervals with 200μg of protein emulsified in Freund's complete adjuvant for the first immunization and incomplete adjuvant for the following three immunizations. Final bleeding was carried out two weeks after the last immunization and total IgG was purified on Protein G agarose columns and subsequently dialyzed and concentrated.

Serum samples

Human serum from individuals living in malaria endemic regions (both asymptomatic adults and symptomatic children) were used in Papers I, II and III and sample collection has been reported elsewhere (Normark et al. 2007; Leitgeb et al. 2011; Nilsson et al. 2011)

Enzyme-linked Immunosorbent Assay (ELISA)

Antibodies (either in human sera samples from individuals exposed or not to malaria and in animals upon immunization with *P. falciparum* recombinant proteins) against recombinant proteins or peptides, were measured by ELISA as previously described (Nilsson et al. 2011). Plates were coated overnight at 4°C with peptides or recombinant proteins. Plates were washed and then blocked with 1% bovine serum albumin (BSA) followed by three washes. Bound IgG was measured by incubation for one hour at room temperature with alkaline phosphatase-conjugated antibodies diluted in PBS. Plates were washed three times and developed with SigmaFast p-nitrophenyl phosphate tablets. The optical density (OD) was measured at 405 nm in an ELISA reader.

pRBC surface reactivity measured by flow cytometry

Antibody binding to pRBCs was tested using flow cytometry as previously described (Albrecht et al. 2011). Briefly, the pRBCs were blocked for 1 hour with 2% fetal bovine serum (FBS) in PBS followed by incubation with a primary antibody (monoclonal/polyclonal IgG or serum) for 30 min at room temperature. The pRBCs were washed three times with 2%FBS in PBS followed by incubation for 30 min at room temperature with an appropriate secondary antibody coupled to Alexa488 (Molecular Probes®, Life Technologies, dilution 1:200) and ethidium bromide/Hoechst or DHE to stain the parasite nuclei. Finally the pRBCs were washed three times with 2%FBS in PBS followed by flow cytometry analysis. Results were expressed either as geometric mean fluorescent intensity (MFI) or as percentage of positive pRBC.

Rosette disruption assay

The ability of antibodies to disrupt rosettes was tested as described before (Treutiger et al. 1992; Ch'ng et al. 2016). Antibody samples were tested in 50µl of parasite suspensions, at 5% hematocrit and at least 5% parasitaemia. The samples were incubated in duplicates at room temperature for 1 hour, followed by parasite staining with acridine orange or Hoechst/DHE and counting of rosettes under the microscope or by flow cytometry (percentage of multiplets as a rosette percentage readout). For microscopy counts at least 200 pRBCs were considered and the rosetting rate in the presence of antibody was calculated relative to the rosetting rate in the negative control (no serum added).

Phagocytosis assay

The phagocytosis assay used in papers II and III was performed as described before (Ghumra et al. 2011) with modifications. The human monocytic line THP-1 was used. Cells were checked periodically for surface expression of the Fcγ-receptors I (CD64) and II (CD32) on the surface, necessary for the antibody-dependent phagocytic activity (Fleit & Kobasiuk 1991; Auwerx et al. 1992).

Synchronized and purified (using a VarioMACS magnet) FCR3S1.2 pRBCs (30-32 hpi, 10% parasitaemia and >70% rosetting) were used for the phagocytosis assays. The pRBCs nuclei were stained with ethidium bromide solution and incubated in the appropriate antibody concentration solution. A positive (rabbit anti human red blood cells, ab34858, ABCAM, 1:100 dilution) and a negative control (unopsonized control) were always included. The opsonization was performed at 37°C for 45 minutes, followed by three washes with MCM and re-suspension in THP-1 cells culture medium. THP-1 cells were incubated with the opsonized pRBCs for 40 minutes at 37°C, 5% CO_2. The phagocytosis was stopped by centrifugation at 4°C followed by re-suspension in room temperature ammonium chloride lysing solution to lyse non-ingested pRBCs. The lysis was stopped by addition of PBS supplemented with 2% FBS, followed by three washes. After the final wash the cells were analyzed by flow cytometry, gating for THP-1 cells and determining the percentage of ethidium bromide positive cells. The phagocytosis rate was calculated relative to the percentage of ethidium bromide positive cells in the positive control.

Antibody epitope mapping on a peptide array

This approach was used in Papers I, III and IV. The technique was used with two main purposes: (1) to map the specific epitopes recognized by different antibody/serum samples and (2) to check for antibody specificity and cross-reactivity against other proteins (protein families). Two different custom designed arrays were used. In Paper I peptide arrays manufactured by JPT (JPT Peptide Technologies) were used. The arrays included around 100 peptides with a 15 amino acid length covering the sequence of seven different NTS-DBLα domains. In Papers III and IV ultra-dense peptide arrays manufactured by Roche-Nimblegen were used for epitope mapping as described before (Forsström et al. 2014). The array included 175,000 peptides with a 12 amino acid length and an 11-residue overlap. The peptides represented the sequences of several *P. falciparum* surface antigens, including the 2TM family, PHISTs, RIFINs, STEVORs, SURFINs and a handful PfEMP1s.

Immunoblot analysis on *P. falciparum* material

In order to test SURFIN$_{4.2}$ temporal expression during the asexual cycle inside RBCs, parasite protein extracts were prepared as described before (Cooper 2002). Briefly, total parasite culture was collected, medium removed and parasite pellet treated with 0.01% saponin solution in PBS. The suspension after lysis was centrifuged and the pellet further extracted in a 2% detergent solution plus protease inhibitors (Roche, Complete EDTA free tablets). The zwitterionic detergent SB3-10 (3-(Decyldimethylammonio) propanesulfonate inner salt) was routinely used after an initial detergent screening and also due to its reported efficiency to extract membrane associated proteins under non-denaturing conditions (Everberg et al. 2006). The soluble fraction after detergent extraction was used to run both SDS-PAGE and blue native (BN)-PAGE. During the initial screening the pellet was re-extracted in SDS 2% in PBS, to assess protein extraction efficiency into the soluble fraction. After electrophoresis proteins were transferred to nitrocellulose or PVDF membranes and used for immunoblot.

Merozoite purification

Viable merozoites were purified as described before (Boyle et al. 2010). Briefly, tightly synchronized parasites were purified on a magnetic column when they reached early-segmented schizont stage. After purification, the E-64 protease inhibitor was added at a final concentration of 10µM. After incubation for 5-8 hours and when segmentation was complete (with clear fully mature sacks of merozoites observed), cells were collected by centrifugation, re-suspended in medium without serum and filtered through a 1.2µm syringe filter. Flow through after filtering was either used directly for imaging of the invasion process adding it to RBCs as described before (Riglar et al. 2011) or re-centrifuged to obtain a merozoite pellet for protein extraction.

Immunofluorescence assay (IFA)

To assess protein localization during the asexual cycle parasites were collected at different time points and prepared for fluorescence microscopy. Culture medium was removed and cell pellet washed three times with PBS. Microscope slides were treated with 0.1% Poly-L-Lysine and after a wash with PBS cell suspension was added and incubated in a humidified chamber. Cells were fixed in 3% paraformaldehyde in PBS, followed by a 10-minute permeabilization step in 0.1% Triton X-100 (for some experiments this step was omitted). Bound cells were blocked overnight followed by incubation with primary antibodies. After thorough washing secondary antibodies coupled to a fluorophore were added. Finally, after washing the secondary antibody a few drops of mounting media with DAPI were added followed by sealing with a coverslip.

Immunoelectron microscopy (iEM)

In order to establish in fine detail the $\text{SURFIN}_{4.2}$ localization in the merozoite stages, purified merozoites were fixed in 3 % paraformaldehyde in 0.1 M phosphate buffer. After fixation cells were washed and embedded in 10% gelatin. Samples were then infiltrated into 2.3 M of sucrose and frozen in liquid nitrogen. Sectioning was performed at -95°C and placed on carbon-reinforced formvar-coated, 50 mesh Nickel grids. Immunolabelling procedure was performed as follows: grids were blocked in 2% BSA and 2% Fish gelatin in PBS. Sections were then incubated with the primary antibody and thoroughly washed. Antibody binding was detected with protein A coated with 10 nm gold. Sections were rinsed and fixed in 2% glutaraldehyde contrasted with 0,05% uranyl acetate and embedded in 1% methylcellulose. Preparations were examined in a Hittachi 7700 and images acquired with a Veleta camera.

Culture supernatant preparation

In order to test if $\text{SURFIN}_{4.2}$ was being shed into culture supernatant upon schizont rupture, FCR3S1.2 synchronized culture was grown till parasites reached early schizont stage followed by purification on a magnetic column. The purified schizonts were allowed to grow and egress in the absence of human serum and RBCs. Culture supernatants were collected after two steps of centrifugation. First centrifugation was done at 3300g for 15 min at 4°C to remove schizonts and free merozoites. Supernatant was then further ultracentrifuged and pellet and supernatant (before and after ultracentrifugation) were analyzed on SDS-PAGE followed by immunoblot with anti-$\text{SURFIN}_{4.2}$ antibodies.

Immunoprecipitation (IP) and mass spectrometry (MS)

To assess potential $\text{SURFIN}_{4.2}$ interacting partners, IP with anti-$\text{SURFIN}_{4.2}$ antibodies followed by protein identification by MS was employed. Parasite protein extract was prepared as described (saponin followed by SB3-10 extraction) and IP was performed using a commercial kit following the manufacturer´s instructions. Eluted fractions after IP were run on SDS-PAGE, transferred into nitrocellulose membrane for immunoblotting.

For MS, the elution fraction after IP with anti-$\text{SURFIN}_{4.2}$ and control antibodies was run on SDS-PAGE, followed by staining with colloidal blue. Fragments where protein bands were clearly observed were sent for MS protein identification to alphalyse (www.alphalyse.com) following company's standard procedures.

Growth inhibition assay (GIA)

Synchronized parasites were grown till they reached trophozoite stage, then a suspension at 0.5% parasitaemia and 5% hematocrit was prepared in the presence of anti-SURFIN$_{4.2}$ or control antibody solution at different concentrations. After one cycle of re-invasion final parasitaemia was measured using flow cytometry after acridine orange staining. Parasitaemia was calculated as a percentage of that obtained with a control, were PBS was added instead of antibody.

Analysis

Flow cytometry analysis was performed using the FlowJo version 9.2 software (TreeStar, USA). Mean, standard deviations (SD) and figures were performed using the GraphPad Prism version 6.0f for Mac OS X (La Jolla, California, USA). All values are expressed as mean ±SD from 3 independent experiments.

4 RESULTS AND DISCUSSION

4.1 PAPER I

Anti-rosetting monoclonal antibodies recognized epitopes localized in Subdomain-3 of the NTS-DBL1α

As mentioned in the introduction, rosetting has been correlated with severe malaria (Carlson et al. 1990; Treutiger et al. 1992; Rowe et al. 1995; Roberts et al. 2000; Normark et al. 2007) suggesting this is a parasite phenotype contributing to the disease pathogenesis. Potent anti-rosetting antibodies have been generated upon animal immunization with recombinant PfEMP1 domains (Ghumra et al. 2011; Vigan-Womas et al. 2008; Vigan-Womas et al. 2011), however the specific target and the molecular mechanisms underlying the disruption were not clear. In this study we decided to approach this gap in knowledge by producing a set of monoclonal and polyclonal antibodies against three recombinant NTS-DBL1α domains expressed by three different parasite strains characterized by their rosetting phenotype. Antibodies were initially tested for their ability to recognize the recombinant proteins (by ELISA) as well as the native protein expressed on the pRBC surface. More importantly we tested for the antibodies' ability to disrupt rosettes formed by the homologous parasite.

After assessing these characteristics, we could divide the antibodies in three groups based on their ability to recognize the surface and disrupt the rosettes. The first group included antibodies that were able to both recognize the surface and disrupt rosettes (Table 1, depicted in red and green). The second group included antibodies that recognized the surface but did not disrupt rosettes (Table 1, depicted in blue). The third group did not recognize the pRBC surface and did not disrupt rosettes (Table 1, depicted in black). By using a peptide array covering the entire sequences of the domains used for the animal immunization, we were able to map the epitopes recognized by most of these antibodies. Antibodies belonging to the first group (surface reactive and rosette disrupting) recognized a sequence of amino acids localized on the loop connecting helix 6 and helix 7 of the DBL1α subdomain 3 (SD3) (Fig. 9), we designated this region as the SD3-loop region. The consistent targeting of this particular region suggests this is an immunodominant region of the protein that is in some way involved in rosette formation. For a few antibodies within this group (mAbV2-7, mAbV2-13, mAbV2-14.1, mAbV17.1) we could not map the epitopes on the peptide array, suggesting they recognize conformational epitopes available on the folded native protein presented on the pRBC surface. However a few of them (mAbV2-7, mAbV2-13, mAbV2-14.1) recognized longer versions of the SD3-loop (19 amino acids long versus 15 used on the peptide array) when tested by ELISA. We speculate the extended peptide used for ELISA probably resembles better the epitope presented on the pRBC surface. For the second group of antibodies (surface reactive but non rosette disruptive), we could not map the peptides, neither on the peptide array nor on ELISA over longer peptides, indicating again they recognize conformational epitopes, only present on the native protein located at the pRBC surface. The third group of antibodies (non-surface reactive and non-rosette disruptive) recognized a region in the middle of the SD3-loop (mAbV2-c21), a region in the beginning of helix 7 (mAbV2-c20 and mAbR29-c3) and a region on SD2 (mAbR19c-4), all inaccessible on the native protein presented on the pRBC surface.

	Immunogen (NTS-DBL1α)	Antibody type	ELISA reactivity	Surface reactivity	Rosette disruption	Phagocytosis	Peptide array reactivity
mAbV2-7	ITvar60	IgG2a	+	+	+	+	SD3-loop**
mAbV2-11*	ITvar60	IgG2a	+	+	+	n.d.	SD3-loop
mAbV2-13	ITvar60	IgG2a	+	+	+	+	SD3-loop**
mAbV2-17.1	ITvar60	IgG2a	+	+	+	+	-
mAbV2-3	ITvar60	IgG2b	+	+	+	-	SD3-loop
mAbV2-6	ITvar60	IgG2b	+	+	+	-	SD3-loop
mAbV2-14.1	ITvar60	IgG2b	+	+	+	n.d.	SD3-loop**
mAbV2-14.2	ITvar60	IgG2b	+	+	+	-	n.d.
mAbR29-1.1*	ITvar9	IgG2b	+	+	+	n.d.	SD3-loop
mAbV2-4	ITvar60	IgG2a	+	+	-	+	-
mAbV2-16	ITvar60	IgG2a	+	+	-	+	-
mAbV2-c20	ITvar60	IgG2a	+	-	-	n.d.	SD3-H7
mAbV2-c21	ITvar60	IgG2b	+	-	-	n.d.	SD3-loop (YCSGDG)
mAbR29-c3	ITvar9	IgG2a	+	-	-	n.d.	SD3-H7
mAbR29-c4	ITvar9	IgM	+	-	-	n.d.	SD2 (LARSFADIG)
Sly D	-	IgG	-	-	-	-	-
ITvar60 goat	ITvar60	IgG	+	+	+	-	+
ITvar9 goat	ITvar9	IgG	+	+	+	-	+
varO goat*	PAvarO	IgG	+	+	+	n.d.	+
Control goat Ig	-	IgG	-	-	-	-	-
Rat SD1*	SD1 ITvar60	IgG	+	+	-	n.d.	n.d.
Rat SD2*	SD2 ITvar60	IgG	+	+	-	n.d.	n.d.
Rat SD3*	SD3 ITvar60	IgG	+	+	+	n.d.	n.d.
IM1	Natural exposure	Serum	+	+	-	+	n.d.
IM2	Natural exposure	Serum	+	+	-	+	n.d.
IM3	Natural exposure	Serum	+	+	-	+	n.d.
IM4	Natural exposure	Serum	+	+	-	+	n.d.
IM5	Natural exposure	Serum	+	+	-	+	n.d.
IM6	Natural exposure	Serum	+	+	+	+	n.d.
IMP	Natural exposure	Serum	+	+	-	+	n.d.
SCP	-	Serum	-	-	-	-	n.d.

Table 1. Summary table of the antibodies characterized in **Papers I and II**. Antibodies are color-coded based on their ability to disrupt rosettes and induce phagocytosis (in red), only disrupt rosettes (in green) and only to induce phagocytosis (in blue). * These antibodies were assigned to the different groups based on their isotypes/species origin and behavior on surface reactivity and rosette disruption despite the fact they were not tested for phagocytosis induction. ** Conformational epitopes tested on ELISA over longer SD3-loop peptide. n.d. not determined

To further verify the importance of the SD3-loop region, we also tried to map the epitopes recognized by the polyclonal antibodies. In this case most of the reactivity was localized on the NTS with a few peaks along the SD2 and the SD3, one of them being on the SD3-loop region. When the polyclonal antibodies were pre-absorbed over an SD3-loop peptide column, all the reactivity (measured by ELISA) towards this particular region was abolished together with most of the rosette disrupting capacity. We also immunize animals with each separate SD and only antibodies produced after SD3 immunization were able to both recognize the surface and disrupt rosettes, again confirming the importance of these region.

In order to assess if antibody response towards the SD3-loop is achieved during natural exposure to the parasite, sera from individuals residing in endemic areas were tested on

ELISA for recognition of three different SD3-loop sequences, confirming the presence of antibodies targeting this region, suggesting this is a common target of naturally acquired antibodies.

Once we identified the SD3-loop as the region targeted by rosette-disrupting antibodies we wondered if particular sequences of this region were associated with a particular malaria clinical presentation or with a particular parasite phenotype, especially with rosetting. Since most of the sequences available from wild isolates do not include this region, we decided to retrieve sequences from laboratory strains (3D7, IT4, Dd2 and HB3) to build neighbor joining distance trees based on alignments of the different SDs. Surprisingly, trees based both on NTS-SD1 or SD3 alignments shared the grouping pattern. Two clearly defined clusters grouping sequences containing 2 or less cysteines (named group A) or more than 3 cysteines (named group B) were observed. It has been previously reported that PfEMP1s containing 2 cysteines in this region of the sequence are correlated with the ability to form rosettes and also seem to be overrepresented in parasites causing severe malaria (Kirchgatter & Portillo 2002; Bull et al. 2005; Normark et al. 2007). The fact that both regions generate similar grouping indicate that both of them carry sequence features associated with this seemingly functional grouping. Moreover, sequences corresponding to the PfEMP1s expressed by the rosetting parasites FCR3S1.2, R29 and PAvarO fall into the group A cluster.

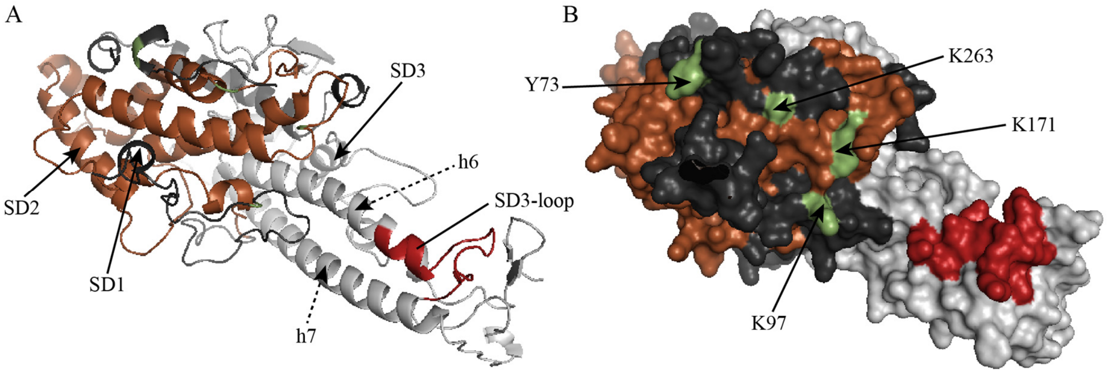

Fig. 9. A. Molecular model of the NTS-DBL1α domain (IT4var60) indicating the different subdomains and showing the SD3-loop region connecting helixes 6 and 7 on SD3. B. Surface molecular model of the NTS-DBL1α domain (IT4var60) showing the SD3-loop region in red and amino acids important for RBC receptor/heparin binding in green.

The receptor-binding site for DBLs involved both in invasion and rosetting has been mapped to central regions of the domain both in SD1 and SD2 (Mayor et al. 2005; Mayor et al. 2009; Juillerat et al. 2011; Vigan-Womas et al. 2012; Angeletti et al. 2015; Guillotte et al. 2016). In Fig. 9B, the different residues identified in the IT4var60 variant as important for RBC/heparin binding are shown (in green) as well as the SD3-loop (in red). This makes evident that both regions are spatially separated. We speculate the targeting of the SD3-loop by rosette-disrupting antibodies could generate a conformational change on the distant binding site impairing interaction with the receptor on the pRBC surface.

4.2 PAPER II

Immunization with an NTS-DBL1α domain induce opsonizing antibodies able to trigger phagocytosis

In **Paper I** a panel of antibodies (monoclonal and polyclonal) against rosette-mediating NTS-DBL1α domains was produced by animal immunization. The antibodies were analyzed with particular attention to their capacity to recognize the surface of the pRBC and disrupt the rosettes formed by homologous parasites. We also successfully mapped on a peptide array the specific epitopes recognized by the majority of these antibodies. Paper II was a follow up study, where we wanted to address the ability of these antibodies to perform an additional effector function that has been previously correlated with protection, opsonization of the pRBC for phagocytosis. This was of particular interest in the cases where pRBC surface reactivity was detected but no rosette-disruption capacity was observed (mAbV2.4 and mAbV2.16).

To assess the capacity of the antibodies to opsonize and induce pRBC phagocytosis, the human monocytic cell line THP-1 was used due to its documented phagocytic activity (Daigneault et al. 2010). This monocytic cell line express Fcγ-receptors I (CD64) and II (CD32) (Fleit & Kobasiuk 1991; Auwerx et al. 1992), both belonging to the activating class that upon recognition of immuno-complexes or opsonized particles/cells trigger signals leading to cellular responses including phagocytosis (Bruhns & Jönsson 2015). The opsonization/phagocytosis assay employed, has been previously standardized (Ghumra et al. 2011) and was used here with minor modifications. In brief nuclei in the pRBCs were fluorescently labeled and pre-incubated with the antibodies (opsonization step) and then mixed with THP-1 cells followed by measurement of the proportion of THP-1 cells that had phagocytosed labeled pRBCs by FACS.

The antibodies tested showed variable abilities to opsonize and induce phagocytosis. As expected, the antibody isotype was a key determinant of the ability to induce or not phagocytosis. Antibodies of the IgG2b isotype did not induce phagocytosis, result explained by the inability of this particular isotype to bind to human Fcγ-receptors (Unkeless et al. 1988). In contrast, antibodies of the IgG2a isotype were efficient at inducing phagocytosis. Interestingly, heterogeneity in the behavior of different antibodies was observed when increasing amount of antibody were used for the opsonization. We described this heterogeneity by grouping the antibodies according to the pattern observed as follows. Antibodies mAbV2-4, mAbV2-7 and mAbV2-16 had a prozone-like behavior, with initial increases in antibody concentration generating increasing levels of phagocytosis, followed thereafter by a decreasing level of phagocytosis upon further increases in the antibody concentration. Antibody mAbV2-17.1 showed a concentration-dependent phagocytosis. Antibody mAbV2-13 was the most potent antibody, reaching high phagocytosis percentages already at low antibody concentrations that did not significantly increase with further increase in concentration.

As observed in **Paper I**, polyclonal antibodies generated in goat were able to recognize the recombinant antigen (measured by ELISA) as well as the native protein expressed on the surface of pRBCs (measured by flow cytometry). More importantly they disrupted rosettes formed by the homologous parasite. However, when they were tested for their ability to induce phagocytosis by the THP-1 cells, not even high antibody concentrations were effective, with the observed phagocytosis being similar to the one for the negative control (total IgG purified from a control goat). This can be explained again by the inability of goat IgGs to bind efficiently human Fcγ-receptors (Micusan & Borduas 1977; Alexander & Sanders 1977).

We also used a set of 6 human sera from Myanmarese immune adults (individually or pooled together) to contrast their phagocytosis ability with that generated after animal immunization. All samples showed the presence of naturally acquired antibodies able to recognize the pRBC surface of the model parasite tested. More importantly these antibodies induced phagocytosis by THP-1 cells. Antibodies (detected by ELISA) were found against the particular NTS-DBL1α variant expressed by the parasite used in all samples, however the pRBCs surface reactivity and the induction of phagocytosis observed, are most likely dependent on the recognition of other targets besides the particular PfEMP1 domain (e.g. other PfEMP1 domains, RIFINs, SURFINs, STEVORs and possibly others). Even though studies have suggested that most of the surface reactivity against pRBCs is due to anti-PfEMP1 antibodies (Marsh et al. 1989; Bull et al. 1998; Ofori et al. 2002; Kinyanjui et al. 2003; Chan et al. 2012), other studies have also suggested the presence of anti-RIFIN and anti-SURFIN$_{4.2}$ antibodies (Abdel-Latif et al. 2002; Abdel-Latif et al. 2004; Kaewthamasorn et al. 2012).

To summarize, in Table 1 surface reacting antibodies are categorized in three groups. A first group included antibodies effective both at disrupting rosettes as well as at inducing phagocytosis (depicted in red, mAbV2-7, mAbV2-13, mAbV2-17.1, possibly mAbV2-11 and one of the human immune samples). As described in **Paper I**, most of these antibodies targeted the SD3-loop region. The second group of antibodies was effective at disrupting rosettes but ineffective at inducing phagocytosis (depicted in green, mAbV2-3, mAbV2-6, mAbV2-14.1, mAbV2-14.2, possibly mAbR29-1.1 and the three goats immunized with different NTS-DBL1α), again most of the antibodies included in this group targeted the SD3-loop. The first and second group main difference resides on their ability/inability to induce phagocytosis and this in turn was linked to the antibody isotype (IgG2a vs. IgG2b) and the origin of the antibody (animal species), that ultimately determined the ability to interact or not with human Fcγ-receptors. The third group included antibodies effective at inducing phagocytosis but not able to disrupt rosettes (depicted in blue, mAbV2-4, mAbV2-16 and most of the human immune samples), for these antibodies the specific epitope(s) (for the antibodies induced upon immunization) and protein(s) (for the naturally acquired antibodies) are not known. For the monoclonal antibodies, is very likely that the epitopes are conformational since we were not able to map them on a linear peptide array. Additionally the results indicated that other regions of the NTS- DBL1α besides those involved in rosetting are exposed on the pRBC surface and are able to induce antibodies with effector functions. Not surprisingly we showed in this study that the most important factors defining the level of phagocytosis are antibody surface reactivity and in general ability of the antibodies to bind phagocytosis-triggering Fc receptors expressed by the effector cell (in this case CD64 and CD32 on THP-1 cells), independently of the ability to disrupt rosettes.

4.3 PAPER III

Naturally acquired antibodies have limited access to the pRBC surface in rosettes formed with blood group A RBCs

As mentioned above and in **Paper II**, we found indications that other regions on the NTS-DBL1 α besides those involved in rosetting were exposed on the pRBC surface and were able to induce antibodies with effectors roles that could be protective in the context of clinical and severe malaria development (specifically opsonization for phagocytosis). Also based on our own evidence and in previous studies, we believe that naturally acquired antibodies during exposure to the parasite, target other proteins on the pRBC surface besides PfEMP1. We tried to verify these possibilities assessing the presence of antibodies against three parasite-derived pRBC surface proteins (PfEMP1, RIFIN-A and SURFIN$_{4.2}$) in sera collected from children suffering from mild or complicated malaria. Seroprevalence and antibody levels were measured in the samples and the values were contrasted to the antibodies present in Swedish adults control sera from individuals that have not been exposed to malaria. In addition, different variables were also measured in the presence of these sera samples, namely, rosetting rate, surface reactivity and opsonization for phagocytosis on the rosetting parasite FCR3S1.2 grown in group O or group A RBCs. Correlations of these variables and the total IgG responses against the three surface proteins were also assessed.

The seroprevalence of antibodies against the three proteins was between 29-41% among all the samples, demonstrating the presence of naturally acquired responses upon *P. falciparum* infection against the three proteins. No indication of a significant association between seroprevalence and protection against complicated disease was found, with seropositivity for each antigen being similar regardless clinical presentation. This was surprising since previous studies have suggested an association between low antibody responses against variant surface proteins and severe malaria (Tebo et al. 2002). When prevalence for the other variables measured (surface reactivity, rosette disruption capacity and phagocytosis induction) was contrasted between mild and complicated samples, no association was found. Also when the actual average values for each variable were compared between the two groups, only the percentage of group O multiplets showed a decrease after incubation with sera belonging to the mild malaria group. This suggests that sera from children suffering from mild malaria have antibodies that are able to disrupt slightly more efficiently rosettes formed in group O RBCs. However, since the difference between the percentage of multiplets was only around

1.4% (which translates in an approximate rosette disruption capacity of only 5%), it is difficult to say if this could have any impact on the clinical outcome.

In order to address the functionality of the antibodies measured by ELISA, their surface reactivity (measured as percentage of IgG positive pRBCs) and their capacity to reduce the rosetting rate (measured by flow cytometry as percentage of multiplets) was measured. Surface reactive antibodies were more common when tested in parasites grown in group O RBCs, indicating that the pRBC surface within a group A rosette is less accessible to the antibodies present in the sera tested as compared to group O rosettes (Fig. 10). When the association between surface reactivity and the IgG levels against the three proteins was assessed, only a low positive correlation was observed with anti $\mathrm{SURFIN}_{4.2}$ IgG levels and only when rosetting rate was measured on parasites grown in group O RBCs.

To determine the levels of rosetting both in parasites grown in group O and group A in the presence of children sera, the percentage of multiplets was measured as described before (Ch'ng et al. 2016). Rosette disruption capacity was modest, being detected in 16% and 13% of the samples when tested on parasites grown in group O and group A RBCs respectively (Fig.10). While the prevalence of antibodies against the pRBC surface was relatively high, the presence of rosette disrupting capacity was in comparison relatively low. This discrepancy could be explained by the existence of many other targets on the surface than those involved in the rosetting phenomenon. It is also possible that antibodies able to disrupt rosettes are not so frequently observed, as suggested in a previous study (Vigan-Womas et al. 2010). Another possibility is that the antibodies present in the sera cross-react poorly with the PfEMP1 rosetting epitopes of the variant (IT4var60) expressed by the model parasite despite the good reactivity measured by ELISA. This is supported by some of the data presented in **Paper I** and in another related study from our laboratory (Angeletti et al. 2013), where cross-reactivity against PfEMP1 at the ELISA level was observed, but there was poor or null cross-reactivity with the native protein expressed on the pRBC surface of heterologous parasites expressing different PfEMP1 variants. The fact that neither the surface reactivity nor the percentage of multiplets were highly correlated to the IgG levels measured by ELISA against the particular PfEMP1 variant expressed by the model parasite FCR3S1.2 also support this explanation. When association between rosetting rate and the IgG levels against the three proteins was assessed, only low negative correlations were observed with anti NTS-DBL1 and $\mathrm{SURFIN}_{4.2}$ IgG levels when rosetting rate was measured on parasites grown in group O RBCs. This was confirmed when data was analyzed as a 2×2 contingency table only for the $\mathrm{SURFIN}_{4.2}$ antigen, suggesting an association between IgG levels for this antigen and the rosetting rate, with increasing levels of IgG against $\mathrm{SURFIN}_{4.2}$ generating lower levels of rosetting. This suggests a potential role of this protein in the rosetting, either as a direct ligand for RBC binding or as an accessory element for rosette formation.

To measure the levels of phagocytosis by THP-1 cells upon opsonization with children sera both on parasites grown in group O and group A RBCs, the percentage of phagocytosis was measured as described for **Paper II**. Phagocytosis induction was prominent, both when tested on parasites grown in group O and group A RBCs, being higher on the first case (Fig.10), indicating again that the pRBC surface is more accessible within a blood group O rosette. When association between surface reactivity and percentage of phagocytosis was assessed, only a low positive correlation was observed between the two variables when samples were tested in group O grown parasites, with increasing levels of surface reactivity associated with higher percentage of phagocytosis.

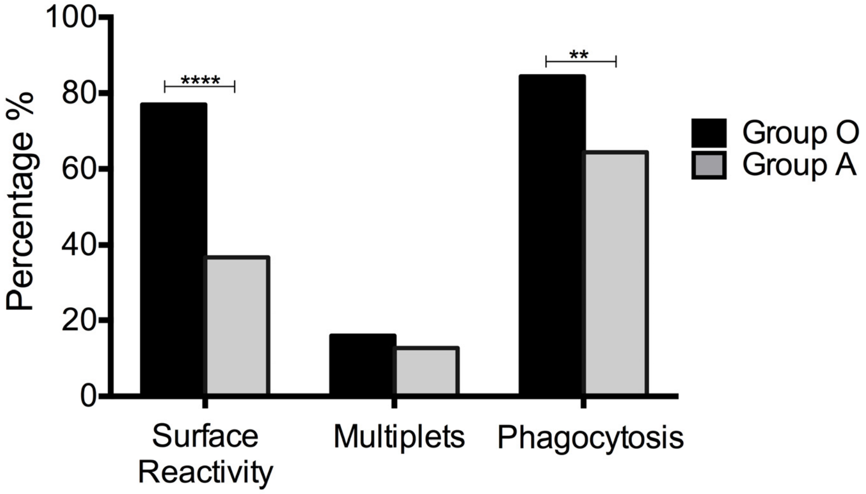

Fig 10. Comparison between percentage of positivity for the surface reactivity, rosette disruption and phagocytosis induction in the presence of children sera when the pRBCs tested were grown in blood group O vs. blood group A RBCs. Differences between the percentages for each group were determined using a chi-square test.

When the average percentages for each variable were compared between group O and group A grown parasites (Fig.11), group O pRBCs again seemed to be more accessible to the antibodies present in the sera tested (higher percentage of IgG positive cells) and were more sensitive to rosette disrupting antibodies present in the sera (lower percentage of multiplets). These findings corroborated published findings from our group showing a decreased accessibility to the surface of pRBCs embedded within a group A rosette (Moll et al. 2015). In this study this is most likely not only limited to the recognition of PfEMP1, but also of other targets on the surface (e.g. RIFIN, STEVOR, $\text{SURFIN}_{4.2}$ and possibly others).

In order to test for particular specificities of the antibodies present in the sera tested against particular regions of different parasite-derived surface proteins that could correlate with the sera ability to perform positively in the assays employed, a small set of samples was selected and tested on a peptide array encompassing the entire repertoire of several reported surface proteins families from 3D7 and IT4 parasites including the three proteins tested here. When samples able to disrupt rosettes of parasites grown in group O RBCs were compared with those that did not, a few peptides differentially recognized were identified. The peptides identified for the PfEMP1 were localized on the DBL1α and 2γ domains. The peptide recognized on the DBL1α was localized by the end of helix 6 and immediately upstream of the SD3-loop, region that was described as the main target of rosette disrupting antibodies generated after animal immunization with this particular domain in **Paper I**.

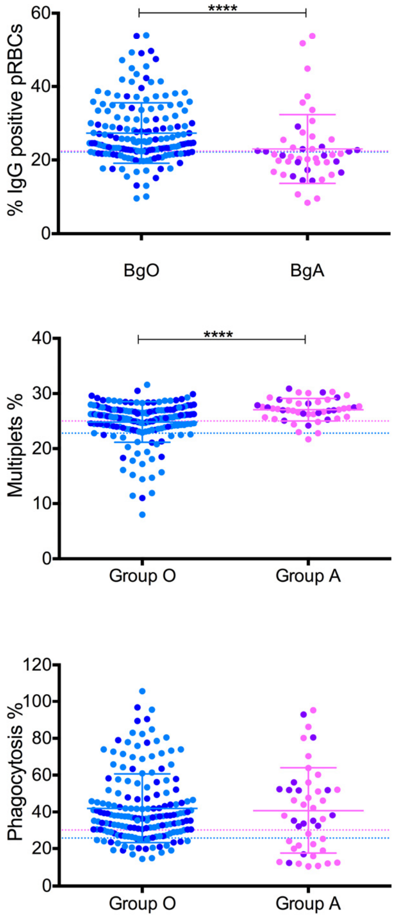

Fig 11. Comparison between the surface reactivity (measured as percentage of IgG positive pRBCs), the rosetting rate (measured as percentage of multiplets) and the percentage of phagocytosis in the presence of children sera when the pRBCs tested were grown in blood group O vs. blood group A. Darker color shades represent samples from children with complicated malaria. Differences between the two groups were determined using a Mann-Whitney unpaired test.

RIFIN-A has been clearly implicated in the rosetting phenotype of the parasite used, however it is not known which particular regions of the protein are involved on RBC binding and which part of the protein is targeted by rosette disrupting antibodies (Goel et al. 2015). The results presented here could not identify a particular region targeted by such antibodies in the extracellular domain. The only region identified was on the intracellular segment, region that is not accessible to antibodies and cannot be part of direct interactions with the RIFIN-A receptor on the RBCs during rosette formation. $SURFIN_{4.2}$ has been described as a pRBC surface antigen, however little is known regarding its function. The fact that it is partially co-transported with PfEMP1 on its route to the surface suggests that it could have a role at this particular cellular location (Winter et al. 2005). The results presented here showed this could be the case since all the variables measured (rosetting rate, surface reactivity and phagocytosis induction) were correlated with the antibody levels measured for this antigen. Similarly as with RIFIN-A the peptide array analysis could not identify particular peptides preferentially targeted by samples with rosette disrupting activity. It is possible that regions targeted by rosette-disrupting antibodies are largely conformational and therefore difficult to identify when only linear peptides are being assessed.

4.4 PAPER IV

SURFIN$_{4.2}$ forms a novel protein complex implicated in *Plasmodium falciparum* invasion

Based on previous studies and on the observation made in **Paper III** we decided to study with more attention the SURFIN$_{4.2}$ protein. The initial purpose was to study its role both in rosetting as well as during the invasion process. For this a gene fragment, encoding the full extracellular domain of SURFIN$_{4.2}$ (PfIT_0422600) was codon optimized for expression in *E. coli* and used to produce an N-terminal His-tagged recombinant protein. The purified recombinant protein was used for animal immunization to generate polyclonal antibodies. Antibody specificity was tested on an ultra dense peptide array (same as the one used in **Paper III**), including 175000 peptides (12 amino acids long with 11-residues overlap), covering several reported *P. falciparum* surface antigens families (2TM, PHISTs, RIFINs, STEVORs, SURFINs and a selected group of PfEMP1s members). The antibodies were specific, with all reactivity observed, confined to the extracellular segment and more importantly, most of the reactive peptides belonged to the protein used for immunization with very limited cross-reactivity with peptides belonging to other protein families included in the array.

Protein expression pattern during the 48-hour asexual cycle of the parasite was tested, both on SDS and BN-PAGE. It was observed that protein amount increased with cycle progression, with higher protein expression during the late trophozoite (36 hours) and schizont (44 hours) stages. Processing of the protein was also observed concomitantly with cycle progression, going from one single band being detected during the ring stage (12 hours) to several bands being detected at later stages. More interestingly the protein was shed into the culture supernatant upon schizont rupture, a very common feature of many proteins involved in the invasion process (reviewed by (Cowman et al. 2012)).

When parasite protein extracts were analyzed on BN-PAGE, protein was only observed on the two last time points, corresponding again to the late trophozoite and schizont stages. More importantly the SURFIN$_{4.2}$ protein was observed not only as monomer but also as part of higher molecular weight species, suggesting the protein is part of a complex formed during late stages. To study this possibility in more detail and address the identity of the possible interacting partners, IP with anti-SURFIN$_{4.2}$ antibodies followed by MS on the eluted fractions was performed. Anti-SURFIN$_{4.2}$ antibodies specifically pulled down bands that corresponded with the previously described pattern. MS on the eluted fractions revealed the presence of GLURP and RON-4, indicating they are forming a protein complex together with SURFIN$_{4.2}$. We decided to name this new protein complex SURGE, based on the complex components (**S**URFIN$_{4.2}$-**R**ON-4-**G**LURP compl**E**x). To corroborate these results and the existence of the complex, IP was repeated and eluted fractions probed with antibodies against the two binding partners identified by MS. Anti-SURFIN$_{4.2}$ antibodies also pulled down GLURP and RON-4 as shown by immunoblots probed with specific antibodies against the two proteins.

To validate the previous reported localization of the SURFIN$_{4.2}$ protein during the asexual cycle, parasite samples were collected every 12 hours and labeled with anti-SURFIN$_{4.2}$ antibodies. As reported previously and also as observed by immunoblot, the protein expression increased with cycle progression (Winter et al. 2005). Signal was weak during ring stages (12 hours) and was mostly associated with the PV while trophozoite stages (24 and 36 hours) showed a stronger signal associated again with the PV as well as with vesicles (presumably MC) in the RBC cytoplasm. Schizont stages showed a clear association between the forming merozoites and the SURFIN$_{4.2}$ protein, with the staining clearly observed at the apical end and the surface of the merozoites.

The specific subcellular localization of SURFIN$_{4.2}$ at the apical end of the merozoite was confirmed using both immuno-fluorescence assays (IFA) and immuno-electron microscopy (iEM). Antibodies against SURFIN$_{4.2}$ were used, as well as antibodies against the merozoite surface (MSP-1 and MSP-3) and the micronemes (EBA-175 and AMA-1). Co-labeling of schizonts with SURFIN$_{4.2}$ and the merozoite surface markers MSP-1 and MSP-3, showed significant signal overlapping. Co-labeling with the microneme markers EBA-175 and AMA-1, showed the staining patterns were in close proximity but no complete overlapping was observed.

When SURFIN$_{4.2}$ localization was assessed during early events of the invasion process, the protein was again observed at the surface and more importantly at the apical end of the merozoite during the attachment to the RBC and active invasion into the nascent PV, suggesting a role during these early events of the process. iEM was chosen as an approach to finely determine the SURFIN$_{4.2}$ subcellular localization, due to the clear and easy morphological identification of the rhoptries in EM. SURFIN$_{4.2}$ was clearly observed at the more apical end of the rhoptries, the neck (Fig.12), consistent with the pattern observed by IFA. In many cases, the labeling was observed extruding out from the rhoptries into the extracellular space. The neck of the rhoptry harbors many other proteins, all involved in the invasion process (e.g. Rhs, RON-2,3,4, and 5),

In order to determine if SURFIN$_{4.2}$ was required during the invasion of *P. falciparum*, the ability of anti-SURFIN$_{4.2}$ antibodies to block parasite invasion was assessed. The growth inhibition assay (GIA) was used for this purpose. The two polyclonal antibodies were tested at different concentrations and only one of the rabbit polyclonal antibodies seemed to inhibit growth in a concentration dependent manner, reaching a 20% inhibition at 1mg/ml.

GLURP was described almost 25 years ago, being observed at the PV during schizont stages and on the surface of newly released merozoites and was suggested to form part of a complex at this location (17). RON-4 is a rhoptry neck protein central for MJ formation as part of the RON complex (formed by interactions with RON-2, and 5). The complex is translocated to the RBC membrane and serves as interacting receptor for AMA-1 (via RON-2) contributing to an essential step of the invasion process.

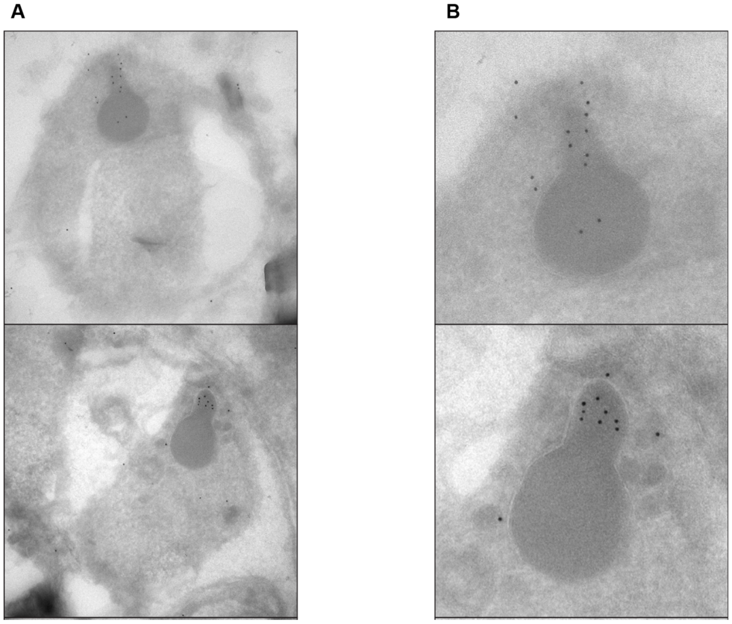

Fig. 12. SURFIN$_{4.2}$ localizes at the rhoptry neck. A. Whole merozoites where the electrodense club-shaped rhoptries are clearly observed. B. Image enlargements of the corresponding pictures shown in A. showing the rhoptries in more detail, SURFIN$_{4.2}$ is clearly present in the most terminal part of the organelle: the neck.

The location of both proteins at the merozoite surface (GLURP) and at the rhoptries neck (RON-4) suggest that proteins may come into contact on the merozoite surface (SURFIN$_{4.2}$-GLURP) and at the neck of the rhoptries (SURFIN$_{4.2}$-RON-4), as well as when the three are shed and/or discharged during invasion. How the three proteins interact both with the parasite membrane as well as with RBC membrane as part of SURGE is not clear and need further study, but we hypothesize this complex is confined to the MJ and contributes to the RBC binding through the N-terminal segment of SURFIN$_{4.2}$.

5 CONCLUDING REMARKS AND FUTURE PERSPECTIVES

A few concluding aspects and future directions are discussed below based on the papers included in this thesis:

I. Anti-rosetting antibodies generated after immunization with an NTS-DBL1-α domain target the SD3-loop segment, localized in subdomain 3. This recognition is suggested to impair rosetting by changing the conformation of the distant receptor binding pocket. Structural studies in presence/absence of rosette-disrupting antibodies could give information regarding possible conformational changes that could explain the exact mechanism of the rosette-disruption activity mediated by these antibodies. It would be also desirable to explore the possibility to generate broadly-reactive antibodies based on the SD3-loop region that could disrupt rosettes formed by different parasites expressing different PfEMP1 variants. One possibility would be to use sequential immunization with different NTS-DBL1-α domains or designing a single hybrid construct including different SD3-loop regions. A challenge of these approaches, however, is the variability observed in this region, making difficult to choose a few variants that truly cover and represent most of the diversity expressed by all rosetting parasites.

II. Immunization with a PfEMP1 NTS-DBL1-α domain elicit antibodies that not only have a biological role in rosette disruption, and therefore in sequestration blocking, but also effectively induce opsonization for phagocytosis of pRBCs. A role that could be of great importance during pRBCs clearance *in vivo*. Some of the antibodies with high opsonizing activity did not disrupt rosettes, indicating that other epitopes besides those involved in rosetting are exposed on the pRBC surface and are able to induce functional antibodies that could provide protection.

III. The data presented also showed that the acquired immune response developed during natural infection can recognize the pRBC surface and more importanly can induce pRBC phagocytosis and in a few cases disrupt the rosettes formed by an heterologous parasite model. These activities however have limited access to the pRBCs inside a group A rosette where the surrounding RBCs act as a cloak for the pRBCs, impairing the antibodies' ability to recognize targets on the surface and perform their effector function (Fig. 13). The study of naturally acquired responses also suggests that SURFIN$_{4.2}$ previously identified at the pRBC surface could be involved in rosette formation either as a direct ligand or as an accessory element for rosette strengthening. Preliminary results indicate that SURFIN$_{4.2}$ binds RBCs (to a yet unknown receptor), indicating its potential as a direct ligand for rosetting (Fig. 13). Moreover, the intracellular segment containing three WRDs (similar to the PfEMP1 ATS segment and a C terminal segment of Pf332) has been suggested to be a scaffold for anchoring to the RBC cytoskeleton, this could be an additional way of SURFIN$_{4.2}$ contributing to *P. falciparum* cytoadhesive properties. (Fig.13). These possibilities however need to be further experimentally validated.

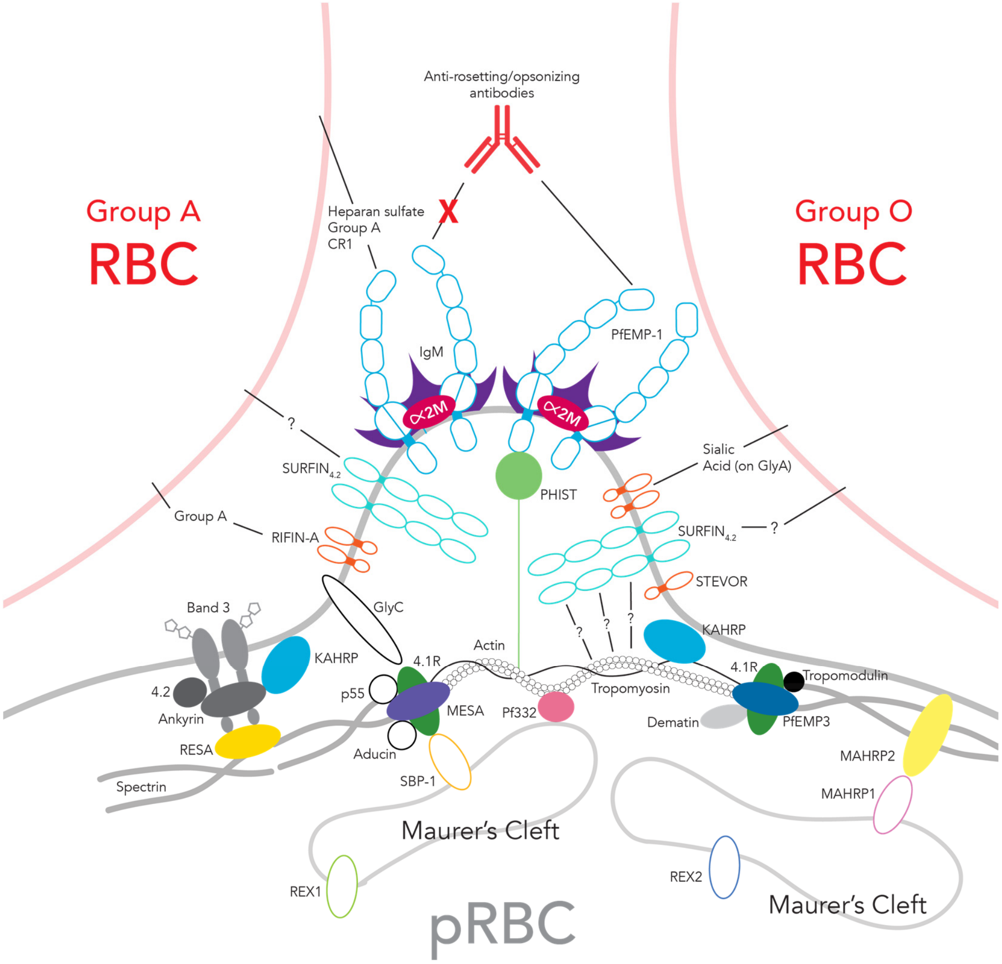

Fig. 13. PfEMP1 and SURFIN$_{4.2}$ location on the pRBC membrane depicting the known binding properties leading to rosetting. The knob structure is depicted indicating most of the underlying protein interactions between parasite-derived antigens (in different colors) and host proteins (in black and shades of gray). SURFIN$_{4.2}$ possible binding capacity with unknown receptors on the RBC and with RBC cytoskeleton elements is depicted. Illustration by Teresa Ducuara.

IV. We also described a novel protein complex involved in *P. falciparum* invasion being SURFIN$_{4.2}$ one of its components. The complex is formed by the interactions between SURFIN$_{4.2}$, GLURP and RON-4 and is possibly localized at the MJ during the invasion process. The precise role of the complex and its topology in relation to the parasite and the RBC membranes requires further study. We hypothesize however that SURFIN$_{4.2}$ is involved in RBC membrane binding through its N-terminal segment and that the complex is confined to the MJ vecinity through its interaction with RON-4 (Fig. 14).

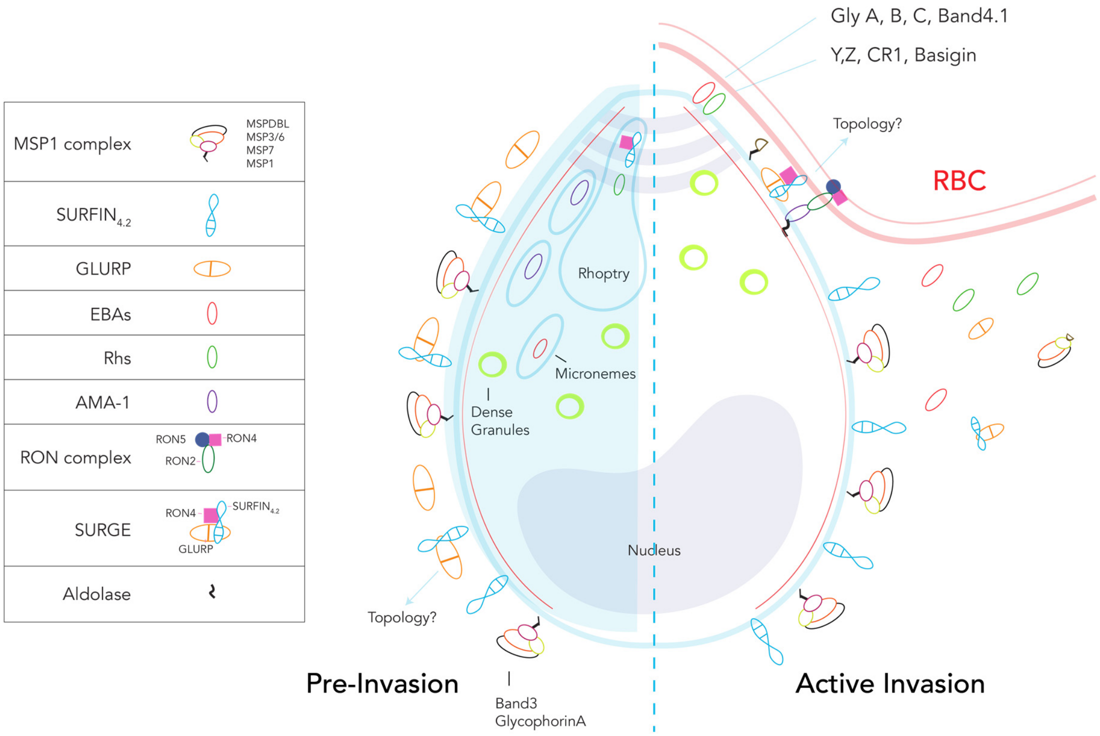

Fig. 14. Schematic representation of a merozoite before (left) and during active (right) invasion. The inferred localization of SURFIN$_{4.2}$ and the protein complexes formed by the protein (with GLURP and RON-4) based on the IFA and iEM are depicted. Illustration by Teresa Ducuara.

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