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EXPLORATION OF FACTORS THAT INFLUENCE *PLASMODIUM FALCIPARUM* FITNESS AND VIRULENCE

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Cover illustration: A *Plasmodium falciparum* rosette in blood by Madle Sirel

EXPLORATION OF FACTORS THAT INFLUENCE *PLASMODIUM FALCIPARUM* FITNESS AND VIRULENCE
THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

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POPULAR SCIENCE SUMMARY OF THE THESIS

Malaria is caused by parasites of the *Plasmodium* family. These parasites spend part of their life cycle replicating inside red blood cells (RBC). Malaria infects 200 million people and kills nearly half a million each year with the heaviest disease burden in sub-Saharan Africa. The disease can vary from asymptomatic to severe. The latter can manifest as impaired consciousness, organ failures and abnormalities in blood or metabolism. Severe malaria is most commonly caused by *P. falciparum*, which is the focus of this thesis. This parasite's extreme harmfulness is in part due to its efficient cell invasion regardless of the age of the RBC and its ability to make parasitized RBC (pRBC) adhere to blood vessel walls. In addition, the infected host cells can bind to uninfected RBCs (uRBC), forming clumps of cells called rosettes. Excessive binding of pRBC in small blood vessels and rosettes can obstruct the blood flow causing oxygen deprivation and damage.

Whether individuals develop severe malaria also depends on human factors. Previous studies have found that blood group O is protective against severe malaria, probably because the rosettes formed in blood group O are smaller and less tight. In **paper I**, we investigated how ABO blood groups affect the parasites ability to form rosettes. Rosettes that are formed in all non-O blood groups shield the parasite better from antibodies. This shielding could hamper the immune clearance of parasites during the infection. We were also interested in understanding how subgroups of ABO blood types affect rosettes. This is because individuals with blood group A can have various levels of A-antigen on the RBC depending on a blood group A subtypes (A1 > A2 > A3 etc.). A positive correlation between A-antigen levels on RBC and the sturdiness of rosettes was seen. In other words, if a person has A1 blood, then the rosettes are more resistant to disruption by antibodies. Therefore, future epidemiological studies investigating risk of severe malaria should keep in mind the subtypes of blood group A.

For the parasites to stick to blood vessels and uRBCs, they need to decorate the surface of pRBC with their own proteins. A member of the parasite protein family called RIFIN has been suggested to bind to A-antigen and mediate rosette formation. There are approximately 200 members in that family and little is known about their function. Many experiments for determining function and location of proteins rely on the use of antibodies against the particular protein. **Paper II** aims to generate and to validate antibodies for the future studies of RIFINs. Antibodies were tested by their ability to recognize a protein that could be a RIFIN based on size, ability to mark the cellular location of RIFINs, and binding specifically to protein fragments of RIFINs. In sum, only a few antibodies performed well in tests and most had specificity issues. Therefore, the antibody should be selected based on the assay in mind.

When the pRBCs block blood flow, the local environment can become acidic due to anaerobic metabolism. In **paper III**, we explored what happens to pRBC stickiness in a low pH environment. We showed that parasites become less adhesive to tissue and form fewer rosettes when pH is altered from what is normal in the circulation. This reduction was linked to the loss of pRBC surface exposed PfEMP1, an antigen that mediates the binding. The total protein

amounts remained similar, but the trafficking to the cell surface or insertion to the membrane was hampered by the drop in pH.

Finally, in **paper IV**, we investigated anti-parasitic properties of a potential new drug, sevuparin. Sevuparin is developed from heparin but lacks heparin's anti-coagulant activity and has been suggested as a promising candidate to be used in combination with other antimalarials. Sevuparin has previously been shown to block parasite invasion into RBCs and inhibit rosetting and cytoadherence. Here, we demonstrate that sevuparin has more modes of action against the parasites. Exposure to sevuparin slows down the development of parasites, reduces multiplication rate, and disturbs pRBC homeostasis. Our results suggest that parasites could be using host membrane channels and transporters for their own advantage. Sevuparin increases the sodium levels in cells, which could explain the observed increase in the lysis of pRBCs upon treatment. We found that sevuparin targets many harmful aspects of the parasite and therefore would be an attractive candidate for adjunctive therapy.

Collectively, the work done here sheds light on factors that affect *P. falciparum*'s fitness and capacity to cause disease. In addition, this work explores the factors that affect the host, the tools that future studies could use, and steps that can be taken to counteract *P. falciparum*'s fitness and capacity to cause disease.

ABSTRACT

Malaria is an ancient disease that still has profound impact on human population. The virulence of the most lethal malaria parasite, *Plasmodium falciparum*, can be attributed to several features of the parasite. *P. falciparum* is known for its indiscriminate red blood cell (RBC) invasion and aptitude for cytoadherence. The latter is associated with various disease pathologies. This thesis explores factors that influence the virulence and fitness of *P. falciparum,* both from the host and parasite perspective.

The association between ABO blood groups and protection from severe malaria has sparked many studies, and blood group O has emerged as protective against severe disease. This protection has been attributed to the binding of uninfected RBCs (uRBC) by the parasitized RBC (pRBC), a mechanism known as rosetting. Using a robust high-throughput flow cytometric method, we characterized rosetting for six parasite strains/isolates in all four major ABO blood groups. Rosettes formed in non-O blood shielded the major parasite surface antigen (PfEMP1) from antibody recognition. As blood group A is further subdivided based on qualitative and quantitative properties of the A-antigen, we found that levels of A-antigen on RBCs were positively correlated with rosette sturdiness against disruption by heparin and antibodies.

RIFINs, another large family of surface antigens, has been implicated in blood group A rosetting. Members of this family can be divided into A- and B-RIFINS, depending on cellular localization and parasite stage expression. To set the scene for future studies of RIFINS, we generated and validated antibodies for various antibody-based methods. We identified two non-rosetting RIFIN-expressing parasite lines that had not been characterized before. Their dominant *rif* transcripts were identified by RNA sequencing.

As PfEMP1s along with RIFINs and other surface adhesins must be trafficked and inserted into the pRBC membrane to fulfil their cytoadhesive function, we hypothesized that his process might be affected by varied conditions in the host. Here, we describe the loss of pRBC's adhesive capacities in acidified environment for rosetting and placental binding parasite stains. The reduction was associated with the loss of surface exposed PfEMP1 due to disturbances in the last steps of PfEMP1 trafficking and membrane insertion.

Heparin-derivatives, including sevuparin, have sparked interest as possible adjunctive therapeutics in severe malaria treatment. Here, we investigated the mechanisms behind the invasion inhibition by clinically well-tolerated sevuparin and explored the additional anti-parasitic properties of this compound. Sevuparin severely affected parasite intracellular development with delayed schizogony and reduced parasitemia after drug removal. The metabolic disturbances manifested in abnormal morphology, abundant extracellular parasites, and reduction of PfEMP1 on the pRBC surface. Inhibition by sevuparin was distinct from classical plasmodial surface anion channel (PSAC) inhibitors, suggesting the involvement of other channels or transporters. Using protein pull-downs from membranes of pRBCs and uRBCs, we identified putative sevuparin interactomes. Due to the identification of multiple

human proteins linked to cation homeostasis and haemolysis, we measured cellular sodium levels. Upon treatment with sevuparin, cellular sodium levels were increased in pRBCs, whereas no differences were noted in uRBCs.

In conclusion, we found that A-antigen levels on RBCs affect rosette characteristics, which should be considered in future studies investigating associations between blood group A and risk to develop severe malaria. We have validated tools for the study of RIFIN family of proteins and their possible function in disease pathogenesis. In addition, we demonstrated that PfEMP1 trafficking to the surface is pH sensitive. Finally, we showed that sevuparin has multimodal activity against malaria parasites.

LIST OF SCIENTIFIC PAPERS

This thesis is based on following papers:

I. Hedberg P, **Sirel M**, Moll K, Kiwuwa MS, Höglund P, Ribacke U, Wahlgren M. Red blood cell blood group A antigen level affects the ability of heparin and PfEMP1 antibodies to disrupt *Plasmodium falciparum* rosettes
Malaria J, 2021 20(1):441

II. Ch'ng JH, **Sirel M***, Zandian A*, Del Pilar Quintana M*, Chun Leung Chan S*, Moll K*, Tellgren-Roth A*, Nilsson I, Nilsson P, Qundos U, Wahlgren M. Epitopes of anti-RIFIN antibodies and characterization of rif-expressing *Plasmodium falciparum* parasites by RNA sequencing
Sci Rep, 2017 7:43190

III. **Sirel M**, Ch'ng JH, Frasch A, Ribacke U. Surface exposure of *Plasmodium falciparum* erythrocyte membrane protein 1 on host cells is pH sensitive
Manuscript

IV. Moll K*, **Sirel M***, Weiss GE, Kodikara CKT, Jonsdottir TK, Végvári Á, Daskalaki E, Habchi B, Wheelock CE, Chan S, Bergquist J, Gilson PR, Ribacke U, Wahlgren M. Sevuparin prevents *Plasmodium falciparum* from deforming and invading red blood cells and hampers intracellular growth by disrupting host cell membrane homeostasis
Manuscript

* Equal contribution

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

I. Rohrbeck L, Adori M, Wang S, He C, Tibbitt CA, Chernyshev M, **Sirel M**, Ribacke U, Murrell B, Bohlooly-Y M, Karlsson MC, Karlsson Hedestam GB, Coquet JM. GPR43 regulates marginal zone B-cell responses to foreign and endogenous antigens.
Immunol Cell Biol. 2021 Feb;99(2):234-243

CONTENTS

LIST OF ABBREVIATIONS

PfATP4	*Plasmodium falciparum* P-type ATPase 4
AP2	Apetala2
ATS	acidic terminal segment
BBB	blood brain barrier
CD36	cluster of differentiation 36
cGMP	cyclic guanosine monophosphate
CIDR	cysteine-rich interdomain
CM	cerebral malaria
DBL	Duffy binding-like
DHODH	Dihydroorotate dehydrogenase
EPCR	Endothelial protein C receptor
ER	endoplasmic reticulum
G6PD	glucose-6-phosphate dehydrogenase deficiency
GYPA	glycophorin A
GYPC	glycophorin C
H3K9me3	histone 3 lysine 9 trimethylation
Hb	haemoglobin
HIV	human immunodeficiency virus
hpi	hours post invasion
HS	heparan sulfate
ICAM1	Intercellular Adhesion Molecule 1
IDC	intraerythrocytic developmental cycle
Ig	immunoglobulin
KAHRP	knob-associated histidine-rich protein
MA-ARDS	malaria-associated acute respiratory distress syndrome
MC	Maurer's clefts
MSP-1	merozoite surface protein 1
ncRNA	non-coding RNA
NO	nitric oxide
NTS	N-terminal segment

OD	optical density
PAM	pregnancy associated malaria
PBS	phosphate-buffered saline
PECAM1	Platelet endothelial cell adhesion molecule 1
PEXEL	*Plasmodium* export element
PfEF2	P. falciparum elongation factor 2
PfEMP1	erythrocyte membrane protein 1
PfMC-2TM	Maurer's clefts two transmembrane
PKG	cGMP-dependent protein kinase
PNEPs	PEXEL-negative exported proteins
pRBC	parasitized red blood cell
PTEX	Plasmodium translocon of exported proteins
PV	parasitophorous vacuole
RAP	rhoptry-associated proteins
RBC	red blood cell
Rh	reticulocyte-binding-like protein homolog
RIFIN	repetitive interspersed protein
RSP-2	rhoptry-derived ring surface protein-2
SERA	serine repeat antigens
STEVOR	Sub-Telomeric Variable Open Reading Frame
SUB1	subtilisin-like protease 1
SURFIN	surface-associated interspersed protein
TNF-α	tumour necrosis factor α
vWF	von Willebrand factor

1 INTRODUCTION

1.1 MALARIA AND GLOBAL HEALTH

Malaria has impacted the lives of humans for thousands of years. Even today it causes substantial morbidity and mortality, threatening the lives of nearly half of the world's population. The disease burden lies heavily upon the sub-Saharan African region (Fig. 1). In 2020, 241 million cases were estimated with 627 000 deaths, with the hardest impact on young children and pregnant women [1]. With this strong impact on humans before reproductive age, malaria has left imprints in human genetics, a phenomenon discussed later in this thesis. The cruelty of this disease not only manifests in the target population but also in the ways it affects the lives of survivors. Malaria has a negative impact on children's education as it often results in absence from school and poorer cognitive performance [2,3]. Moreover, both adults and children suffer from neurological sequelae after recovering from cerebral malaria [4]. In general, morbidity caused by malaria results in days missed at work and hinders economic growth in a region. On the African continent, the disease itself has been proposed to be responsible for a 1.3% reduction in economic growth [2]. Malaria endemicity also leads to loss of investments and tourism [2]. Thus, the disease presents a "Catch-22": elimination of malaria requires resources but malaria itself hinders economic growth.

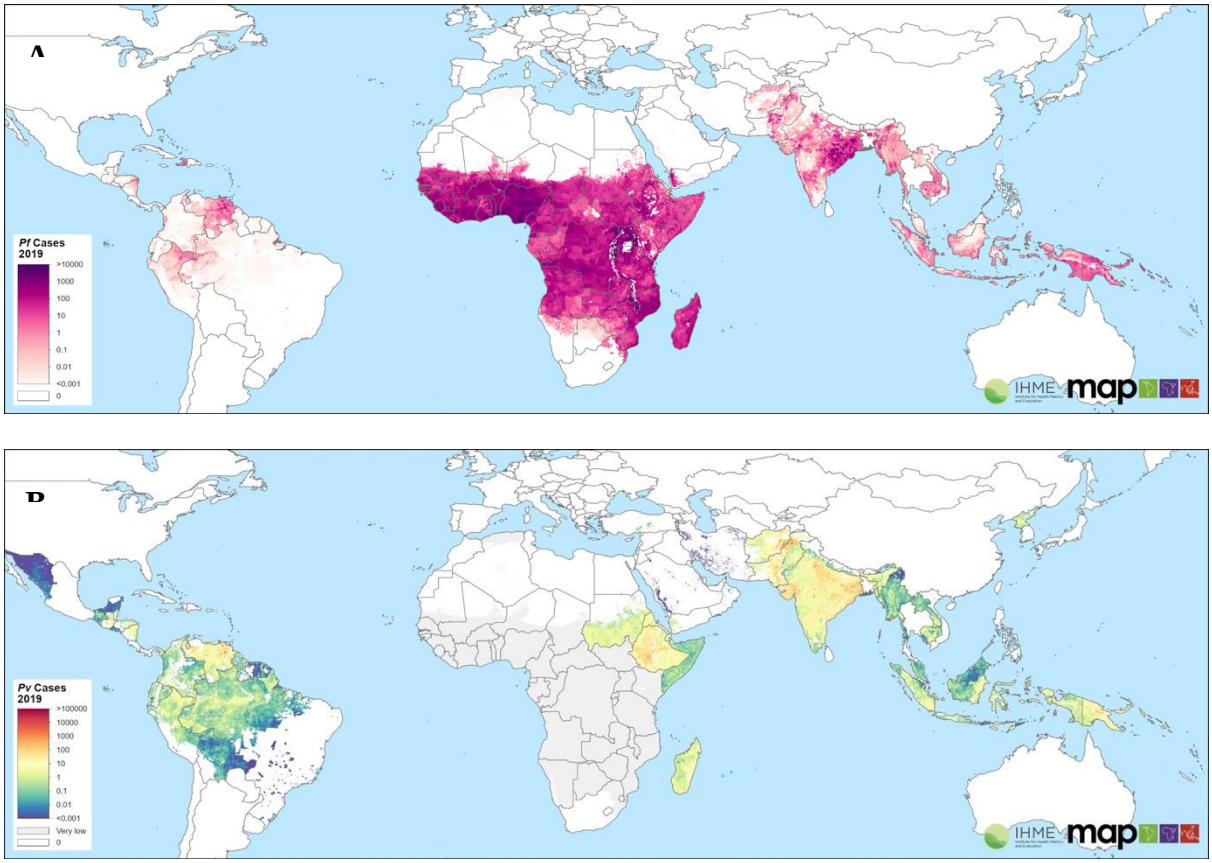

Figure 1. Map of predicted clinical malaria cases in 2019. **(A)** *Plasmodium falciparum*, responsible for the majority of global cases, clinical cases in all age groups. **(B)** *Plasmodium vivax*, accounting for 2% of global cases [1], cases in all age groups. The areas with insufficient data for prediction are light grey. The maps were obtained from the Malaria Atlas Project under the CC BY 3.0.

Several elimination and eradication efforts to combat malaria have been initiated historically, but these efforts have not prevented resurgence of the disease. These resurgences have been intimately linked to socioeconomic factors as active and long-term intervention is needed, which is economically costly [5]. Furthermore, malaria control programs heavily rely on foreign investments, which are influenced by politics and changing priorities. The impact of the COVID-19 pandemic has negatively impacted malaria control efforts. Although the number of COVID-19 cases seems to be lower than expected in Africa [6], approximately two-thirds of additional malaria deaths (compared to 2019) were linked to reduced access to preventive services and other malaria provisions [1]. Besides the socioeconomic factors, malaria elimination and eradication programs have also been compromised by the development of drug resistance among parasites and mosquitoes.

1.2 DRUG RESISTANCE AND NEW INTERVENTIONS (THERAPEUTICS AND VACCINES)

As with any infectious disease, the arms race between the etiological agent and science is continuous. In the case of malaria, many players are involved. To begin with, the successful vector control by indoor residual spraying and insecticide-treated bed nets is jeopardized by development of resistance to pyrethroid insecticides by mosquitos and change in mosquito feeding habits [7–9]. Another reason for the plateauing of malaria elimination progress is the spread of parasite populations with increased tolerance to artemisinin analogues, which are central to current first-line combination therapies, as well as to partner drugs used in combination therapies [10]. Moreover, recently it has been discovered that parasite populations have evolved to escape diagnosis through deletion or drastic changing of genes encoding targets of rapid diagnostic tests [11–13]. Thus, there is a great need for the development of new therapeutic and diagnostic approaches. To delay the development of resistance, new therapeutics should preferably be fast-acting, target several cellular processes, or target host factors required for intracellular parasite growth [10].

On the brighter side, there are at least 13 new antimalarial drugs in clinical development and several of them are against novel targets [14–16]. The parasite's intraerythrocytic growth requires uptake of various nutrients through alterations of host cell membrane permeability (see section 4.2), which presents an attractive process to target. This increased permeability facilitates the influx of sodium into the erythrocyte's cytoplasm. Thus, the parasite needs to actively regulate the Na^+ levels to maintain homeostasis. One of the drugs in the pipeline is Cipargamin (KAE609), which inhibits the P-Type Na^+ATPase (PfATP4) and leads to increased Na^+ levels inside the parasites, resulting in parasite death [17]. Cipargamin is in phase II clinical trials [14,18]. Another drug with a novel mechanism of action is Ganaplacide (KAF156), which is being tested in phase IIb trials in combination with Lumefantrine. Although the exact target is unknown, it is believed to affect parasite internal protein secretory pathways [14,16,18]. A key feature of malaria is the rapid proliferation parasites, which requires frequent rounds of DNA replication. The nucleotides needed for this can either be synthesized *de novo* or salvaged. A rate-limiting step in pyrimidine *de novo* synthesis is the

catalytic activity of dihydroorotate dehydrogenase (DHODH). A selective inhibitor of parasite DHODH, DSM265, is now being investigated in phase II clinical trials [16]. However, there is some concern regarding the ease of resistance development toward DHODH inhibitors [19]. Furthermore, the parasite translation machinery presents yet another promising drug target. Inhibition of *Plasmodium falciparum* elongation factor 2 (PfEF2) by M5717 has shown good efficiency *in vitro* and is now in phase I trials [14,16,20]. As a complement to parasite-killing drugs, adjuvant drugs hampering parasite virulence features such as anti-adhesive polysaccharides are being developed. Sevuparin, an agent manufactured from heparin and that lacks the antithrombin effect, can block parasite invasion to erythrocytes and reverse the binding of infected erythrocyte to uninfected erythrocytes and to the vascular endothelium. Sevuparin was tested in combination with atovaquone/proquanil in phase I/II trials and showed promising results [21].

In addition to new therapeutics, development of an effective malaria vaccine has always been a global goal. In October 2021, the World Health Organization (WHO) endorsed the widespread use of RTS,S/AS01 vaccine [22]. The vaccine is meant for use in children and targets the pre-erythrocytic *Plasmodium falciparum* circumsporozoite protein [23]. However, the efficacy and safety of this four-dose vaccine has been questioned as the protection seems to wane over time [24–26].

Although the future of malaria interventions looks promising, we cannot rest on our laurels and must keep finding new solutions. Therefore, it is important to first increase the understanding of the parasite's biology and pathogenesis of the disease to develop novel targets and to improve the existing combinations.

1.3 MALARIA LIFE CYCLE

This ancient disease is caused by protozoan parasites belonging to the genus *Plasmodium*. The species of malaria parasites causing disease in humans differ in regard to global distribution and life cycle duration. The most prevalent malaria parasite, *P. falciparum*, is responsible for the majority of the disease associated morbidity and mortality and mainly affects sub-Saharan Africa, although it is also found in other malaria endemic regions (Fig. 1A). *P. vivax* is the second most prevalent and is mainly found outside Africa (Fig. 1B). *P. ovale* is widely distributed in tropical and sub-tropical areas, except the Americas, and can be further divided into *P. ovale curtisi* and *P. ovale wallikeri* [27,28]. *P. vivax* and *P. ovale* carry lower risk of fatal outcome and are characterized by tertian fevers (life cycle of 48 hours). *P. malariae*, causing quartan fevers (life cycle of 72 hours) was once probably quite prevalent but is now found in South America, Asia, and Africa but at low frequencies. The sixth malaria parasite, *P. knowlesi*, has zoonotic transmission throughout South East Asia [29]. The life cycle of *P. knowlesi* is the shortest, only 24 hours [30,31].

The life cycle of *Plasmodium* transitions through a variety of morphologically and physiologically distinct stages and requires a human as an intermediate host and an *Anopheles* mosquito as a definite host (Figure 2). The infected female of *Anopheles* mosquito injects

sporozoites into the dermis of the human host while probing for blood. From there, the sporozoites actively make their way into the blood circulation where they are swiftly carried to the liver. In the liver, they invade hepatocytes and forms a parasitophorous vacuole (PV) around them. This phase of the infection is asymptomatic and can last 8 to 25 days depending on the infective species. *P. vivax* and *P. ovale* can arrest growth and establish dormant forms called hypnozoites in the liver, leading to relapsing malaria years after initial clearance of parasites [32,33].

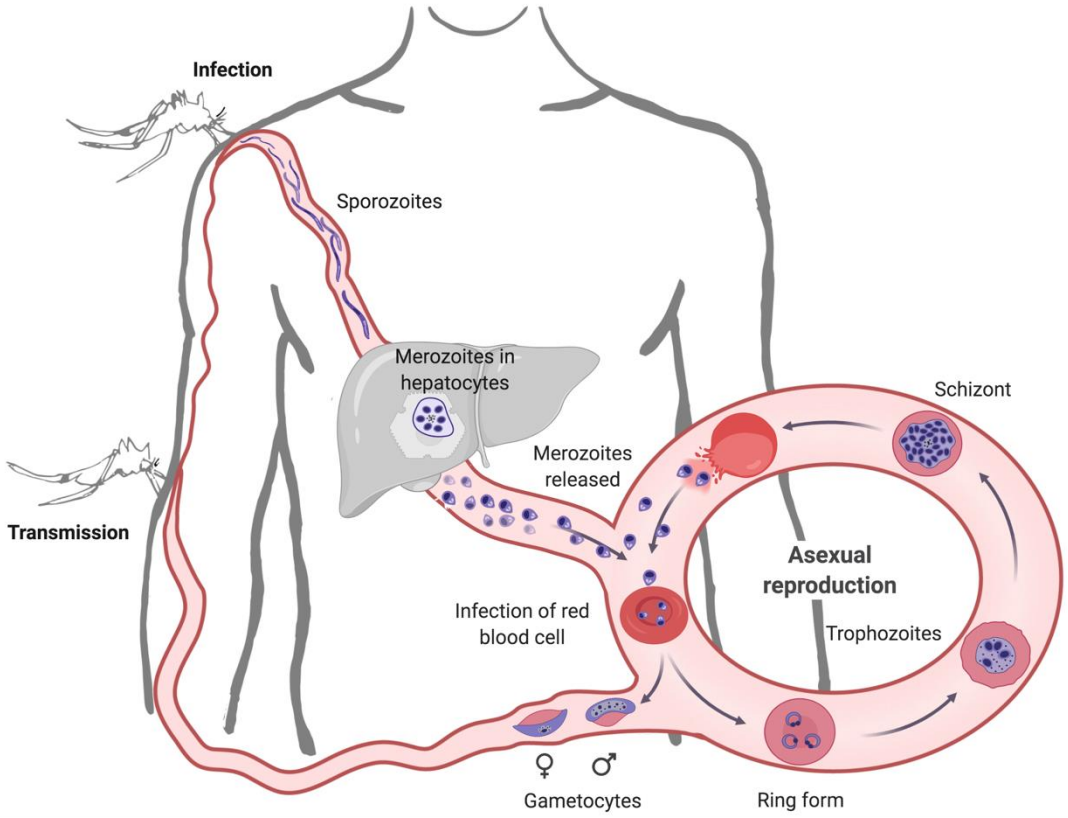

Figure 2. Life cycle of *Plasmodium*. Sporozoites are injected by the female Anopheles mosquito and travel with the blood to the liver, where they establish the initial host cell infection. After several rounds of asexual replication in hepatocytes, the intraerythrocytic developmental cycle begins. Merozoites invade red blood cells and develop into trophozoites, which mature to schizonts and undergo multiple fissions to form new merozoites. A new cycle can begin when the red blood cell ruptures and merozoites are released. A few parasites commit sexually and develop into female and male gametocytes, which can be transmitted back to mosquitos. Sexual replication occurs in the mosquito. Created with BioRender.com.

After the primary replication in hepatocytes, thousands of merozoites are released into the blood stream where they proceed to invade the red blood cells (RBCs). Employing parasite ligands, merozoites attach to specific receptors on RBCs. They actively deform the RBCs and push themselves into the cell via a tight junction (see section 4.1.2). As with the hepatocyte invasion, a PV is formed around the merozoite, within which the parasite can grow and multiply. The following developmental stage is known as ring stage after its ring-like appearance in parasitized RBCs (pRBCs) upon Giemsa staining. As the parasite grows, it changes its shape to a more rounded or irregular trophozoite (about 24 hours post invasion (hpi) for *P. falciparum*). Trophozoites then mature to schizonts (approx. 36 hpi for *P. falciparum*), which undergo mitotic nuclear divisions, finally releasing 16–32 new merozoites during egress

(see section 4.1.1). The cycle repeats with the invasion of new RBCs by merozoites and with an exponential increase of parasite load in the patient. This part of the infection, the intraerythrocytic developmental cycle (IDC), is where clinical manifestations of the disease occur. Each IDC takes 24–72 hours depending on the *Plasmodium* species [32–34].

A fraction of the parasites commits to gametogenesis and become either female or male gametocytes that can be picked up by a mosquito when feeding on blood. After ingestion, male and female gametocytes develop into microgametes and macrogametes, respectively. The flagellated male microgametes fertilize the female macrogamete resulting in a zygote. The zygote transforms into a motile ookinete, penetrates the mosquito midgut wall, and encyst in bodies known as oocysts. Inside the oocyst, parasites divide mitotically and generates a large number of sporozoites. After the oocyst ruptures, the sporozoites migrate to the salivary glands where they are ready to infect a human host again [35].

1.4 *P. FALCIPARUM*'S INTRACELLULAR DEVELOPMENT

Most of the molecular understanding of human malaria parasites comes from research on *P. falciparum*. The following chapter will therefore focus on the *P. falciparum* biology.

1.4.1 Parasite invasion

1.4.1.1 Egress

The IDC ends with a coordinated release of merozoites from pRBC, a process known as egress. What exactly triggers the egress cascade and all the contributing factors remain elusive, but some of the key steps have been identified. Egress begins with rapid accumulation of cyclic guanosine monophosphate (cGMP), which activates the cGMP-dependent protein kinase (PKG) that in return phosphorylates substrates and triggers secondary messenger production [36,37]. Activation of PKG mobilizes the cytosolic Ca^{2+} and leads to rounding up of the PV with merozoites arranged symmetrically in a flower-like manner [38,39]. Occasionally, permeabilization of the PV membrane before rupture has been noted [40]. The combination of Ca^{2+} and PKG activity is required for the discharge and activation of subtilisin-like protease 1 (SUB1) from merozoite exonemes into the PV [41–43]. In the PV lumen, SUB1 cleaves various merozoite surface and PV proteins, such as serine repeat antigens PfSERA5 and PfSERA6, which are crucial in later steps of egress [41,44–47]. Next, the PV membrane ruptures giving the merozoites more freedom to move inside the pRBC [48]. This is followed by the RBC membrane poration and collapse [49]. In the final step of egress, the RBC membrane ruptures, which is believed to be mediated by cysteine proteases as it is blocked by cysteine proteases inhibitor E-64 [50–52]. PfSERA6, one of the proposed enzymes, probably mediates proteolytic degradation of the RBC cytoskeleton [47,50]. The membrane rupture begins from a single point from which a few merozoites are ejected. Thereafter the membrane rips open and curls back, ejecting the remaining merozoites [53].

1.4.1.2 Entry into the new RBC

After egress, merozoites quickly find a new suitable RBC to invade as lingering in the bloodstream might lead to unwanted attention from the immune system. Thus, most invasions occur within a minute after egress and the process itself only requires 20 seconds to complete [54]. The merozoites go through series of events where various parasite proteins and complexes are released in a highly organized manner (Fig. 3).

The initial contact between merozoite and RBC is a weak interaction, possibly mediated by merozoite surface proteins (MSPs) [55]. Of these, MSP1 has been highlighted. MSP1 forms large complexes and some of these bind band 3 and glycophorin A (GlyA) on the RBC membrane [56–58]. However, MSP1 alone does not seem to be essential as parasites lacking surface expressed protein are still able to invade RBCs [59]. Controversially, MSP1 has been proposed to be targeted for invasion blocking by heparin [60,61], but later it has been noted that merozoites can still remain in contact with the RBCs in presence of heparin [54,62]. This controversy could be due to the compensation by other parasite ligands binding to surface of RBCs or that the inhibitory target of heparin is different as heparin has been proposed to bind many different parasite proteins [60].

After the weak initial attachment, merozoite forms high-avidity interactions via adhesins released from the micronemes. Two major protein families have been implicated: Duffy binding-like (DBL) (also known as erythrocyte-binding-like, EBL) proteins and the reticulocyte-binding-like protein homologs (Rh) [55]. Members of these families bind different RBC receptors, for example, EBA-175 interacts with glycophorin A (GYPA) and EBA-140 binds glycophorin C (GYPC) [63–65]. Although none of the proteins individually seem to be essential, their overall function is needed [66]. Furthermore, the diversity in engagement of receptors allows for the use of alternative invasion pathways [67]. These interactions lead to strong deformation of the RBC and reorientation of the merozoite with the apical end towards the RBC membrane. Next, an essential interaction between parasite Rh5 and basigin on the RBC surface occurs [54,68–71]. This event is connected to a calcium flux that is needed for successful invasion and release of further rhoptry content [54]. Among the proteins released are complexes formed in the bulbs of rhoptries: rhoptry-associated proteins (RAP) and a soluble complex composed of the RhopH proteins [72–76]. Among the latter is a small, conserved protein called RhopH3 [77]. Conditional knock-down of RhopH3 reduces the parasite invasive capacity and knockout of this gene kills the parasites [78,79]. Moreover, antibodies against RhopH3 and modifications of the protein inhibit the invasion, but its exact function during the invasion is unknown [74,80,81]. The importance of the RAP complex has been debated as knockdown of RAP1 and RAP2 in *P. berghei* does not affect the invasion [82] whereas knock-down of RAMA, another member of RAP complex, leads to protein mislocalisation and invasion defects [83]. Regardless of the role in invasion, the RAP complex is needed for correct structure of PV membrane and parasite intracellular growth [82].

Next, the RON complex (including RON2, 4 and 5) is injected into the RBC from the rhoptry neck and binds the AMA1 on the merozoite surface to form a tight junction [84–87]. The

invasion begins as the PV forms around the invading parasite. The merozoite pushes through the tight junction propelled by an actin-myosin motor, which is anchored to the tight junction [86,88]. Once inside, the membrane seals behind the merozoite and the PV is pinched off from the RBC membrane [89].

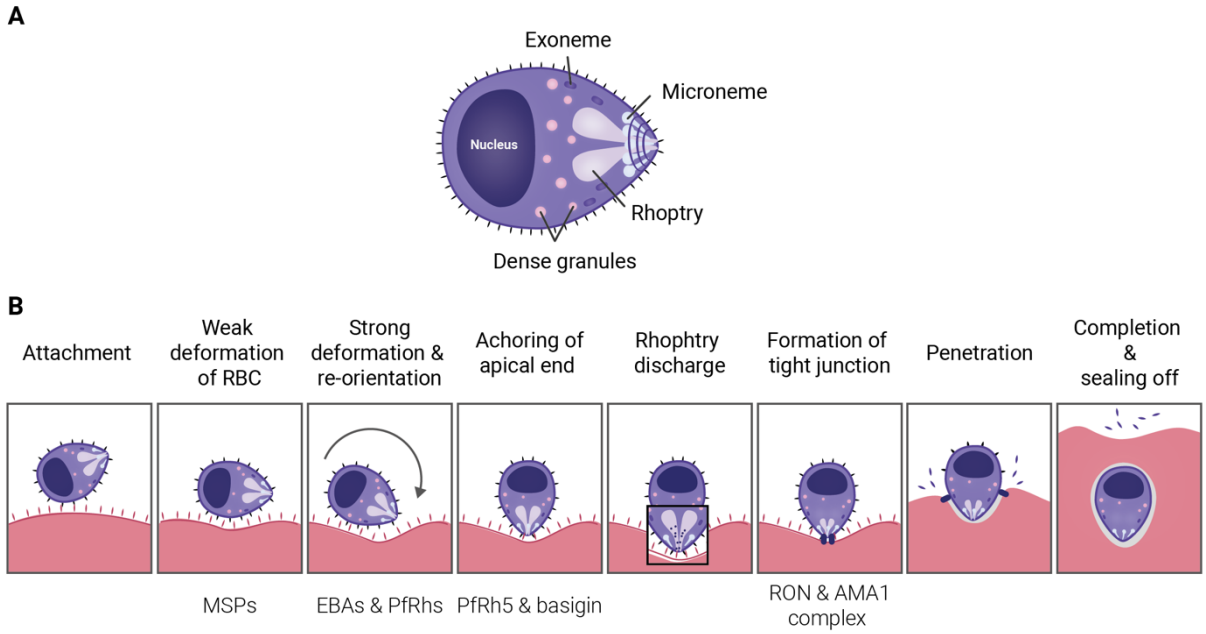

Figure 3. Merozoite invasion. **(A)** Schematic overview of merozoite organelles. **(B)** Steps of merozoite invasion from initial attachment to internalization.

1.4.2 Intracellular development and remodelling

The malaria parasite must establish a suitable environment for growth inside the RBCs, cells that are moderate in their metabolic activity and have lost most of the internal organelles and functional trafficking machineries [90]. Therefore, the parasite needs to remodel the RBC for sufficient nutrient uptake and to perform other necessary functions for survival, such as cytoadhesion.

1.4.2.1 Nutrient acquisition

Growth and multiplication of an organism requires energy and various building blocks. For the malaria parasite, the main source of amino acids is digestion of host haemoglobin, which is internalized via a cytostome [91–93] and degraded by parasite enzymes [94]. However, haemoglobin does not contain all the essential amino acids required by the parasites [95]. For example, it lacks isoleucine and harbours only low levels of methionine. In addition, other nutrients can be rate limiting and need to be scavenged from the plasma, for example, pantothenate, the precursor of coenzyme A and purines [96,97]. Although the RBC membrane contains numerous channels and pumps, not all nutrients can be obtained in sufficient amounts via these mechanisms. Hence, the parasite introduces its own new permeation pathways (NPPs) to import the essential components from the plentiful plasma [98,99]. After infection, the RBC membrane permeability increases drastically to low molecular weight solutes such as sugars, sugar alcohols, organic cations, purines, amino acids,

certain vitamins, and ions [98,100–104]. The best studied and perhaps most important NPP has a strong preference for anion transport over cations and is therefore referred to as the Plasmodium surface anion channel (PSAC) [103,105–107]. NPPs appear on the pRBC surface from 18 hpi to meet the metabolic needs, and their activity peaks around 36 hpi [108]. PSAC is derived from the RhopH invasion complex and is composed of RhopH2, RhopH3, and CLAG [79,109]. These proteins are conserved throughout *Plasmodium* species [110]. RhopH2 and RhopH3 exist as single copy genes [77,111], whereas CLAGs compose a multigene family [112]. CLAG3 has two paralogs that undergo epigenetic switching [113–118], neither of which seem to be essential for parasite growth under standard *in vitro* conditions [119]. This might be because other members of the family can substitute for the function or because of the excess of nutrients in culture medium. However, RhopH2 and RhopH3 are essential for parasite survival, and conditional knock-down causes defects in parasite nutrient uptake [78,79]. Although it's been proposed that other types of parasite derived NPPs exist along-side PSAC, their exact compositions, whether they contain any host-derived proteins and molecules they transport are unknown [120].

Maintaining an appropriate intracellular ion balance throughout IDC is important to avoid premature cell lysis (reviewed in [121]). A by-product of increased permeability to nutrients is the flux of cations via PSAC [105,122]. In uRBCs, the levels of K^+ are kept high and Na^+ low via Na^+,K^+ ATPase, which exports Na^+ and imports K^+. In the pRBCs, the opposite is observed: high levels of Na^+ and low levels of K^+ [123–125]. Initially, Na^+,K^+ ATPase tries to compensate for the excess Na^+ by increasing its activity but soon becomes overwhelmed [108]. Inside the parasite cytosol, the levels of sodium are kept low and the levels of potassium high as in other eukaryotic cells [123,125,126]. *Plasmodium falciparum* P-type ATPase 4（PfATP4) is proposed as the primary mediator of sodium efflux in the parasite plasma membrane, although it is unknown how Na^+ enters the cell or how the potassium levels are maintained [127,128]. Another important cation is Ca^{2+}, which plays a role in various signalling pathways. The levels of calcium are kept low in the uRBCs by Ca^{2+} ATPase, but the levels increase during parasite infection [37,130–133]. Other highly relevant physiological anions are Cl^- and HCO_3^- [121]. Changes in permeability affect their concentrations very little as both are already in electrochemical equilibrium across the RBC membrane. However, a slight increase in Cl^- and HCO_3^- in response to haemoglobin consumption by the parasite has been noted to compensate for the loss of negative charge from the haemoglobin [121,133].

After nutrients have been imported into the pRBC they need to cross two more membranes – the PV membrane and the parasite plasma membrane – before they reach their destination in the parasite cytosol. Solutes smaller than 1400 Da can freely pass the PV membrane via a non-selective channel [134]. The loss-of-function complementation experiments of *Toxoplasma gondii* PV membrane channel mutants suggest that *P. falciparum* protein EXP2 forms this channel [135]. This hypothesis has found further support by the observation that the PV membrane channel frequency correlates with EXP2 expression and mutations in that protein alter channel characteristics [136]. Nutrients reach the final destination – i.e., the parasite cytosol – via variety of parasite transporters on parasite plasma membrane (reviewed in [137]).

1.4.2.2 Protein trafficking

To remodel the RBC and to create the infrastructure for nutrient uptake and waste disposal, the parasite needs to export a wide array of proteins to the PV lumen, to the RBC cytoplasm and to the RBC membrane. The exportome of *P. falciparum* is vast, perhaps containing more than 500 proteins [138–141].

All proteins destined for secretion and export begin with entry to the endoplasmic reticulum (ER) via Sec61 translocons [142]. For recognition, the protein needs to obsess a signal sequence or other suitable signals such as internal transmembrane domains. Proteins destined for PV include a N-terminal signal peptide, which is cleaved upon entry to ER by signal peptidase [143]. The majority of exported proteins have an N-terminal recessed signal sequence and a host targeting signal referred to as the Plasmodium export element (PEXEL) (RxLxE/Q/D) [144,145]. After the recessed signal sequence is cleaved upon entry to ER by plasmepsin V, the PEXEL motif is cleaved and N-acetylated [140,142,146–148]. Although PEXEL processing is essential for the proteins containing the motif, there is a separate group of exported proteins missing this motif and therefore are referred to as PEXEL-negative exported proteins (PNEPs). PNEPs typically have a signal peptide and a transmembrane domain [141]. From the ER, the proteins continue within the secretory vesicles to the PV [149].

Next, the host cell targeted proteins, including PEXEL and PNEPs containing proteins, need to pass the PV membrane [150–152]. This process is mediated by a complex named the Plasmodium Translocon of Exported Proteins (PTEX) [150]. PTEX is built up from three components: a protein unfolding motor, a flange shaped connector, and a PV membrane spanning channel. The first consists of hexamer of heat shock protein 101 (HSP101), which harvests the energy from ATP hydrolysis to unfold the exported proteins [153]. The second part is an adapter feeding the unfolded effector proteins into the membrane channel and is made of seven PTEX150 subunits [153]. Finally, the proteins pass through the funnel-shaped pore formed by a heptamer of EXP2 [153]. In addition, several accessory proteins have been observed interacting with PTEX complex.

In the RBC cytosol, the effector proteins need to be refolded and transported to their final destination. The exact mechanism remains unclear, but the involvement of exported parasite chaperons and co-opted host chaperons has been implicated. Soluble proteins are probably transported through the RBC cytosol by diffusion, whereas proteins containing transmembrane domains or hydrophobic regions are likely to be part of a multimeric complex [154,155]. For example, the parasite encoded co-chaperon HSP40 and chaperon HSP70-x form a complex called J-dots that have been observed in association with the major virulence factor erythrocyte membrane protein 1 (PfEMP1) [156]. Furthermore, HSP40 has been detected to interact with members of PTEX (Hsp101, PTEX150) and with other exported proteins [157]. The next stop for many proteins is Maurer's clefts (MC), which might serve as a concentration and sorting platform for proteins bound for host cell membrane such as the aforementioned PfEMP1 [158]. At the MC, PfEMP1 associates with other proteins such as the knob-associated histidine-rich

protein (KAHRP) [159–161] and is transported to the RBC surface inside the vesicles, which is possibly driven by actin treadmilling [162–164].

1.4.3 Cytoadhesion

One of the major virulence features of *P. falciparum* is its ability to sequester in the microvasculature. The remodelling of the RBCs changes the biomechanical properties of the cell, resulting in rigid pRBCs. As the blood circulates through the splenic sinosoids, any abnormal RBCs, such as rigid pRBCs, are retained and engulfed by the macrophages. To survive, the malaria parasites have developed ways to avoid passing through the spleen: they export ligands to the cell surface to make the pRBCs "sticky" so they can adhere to the endothelial receptors (cytoadhesion) or to other uninfected RBCs (rosetting) (reviewed in [165]). All mature trophozoite stage parasites cytoadhere and this cytoadherence has been linked to various disease pathologies.

For many years, the ability of laboratory parasite strains and clinical isolates to bind two or more uRBCs (i.e., rosetting) has been observed [166–168]. Although the *in vivo* advantage of rosetting is elusive, it has been associated with disease severity in Africa [169–172]. One hypothesis has proposed that rosetting facilitates invasion by providing fresh uRBCs close-by; however, there is no definite conclusion on the matter as there are supportive [173] and confuting findings [174–176]. Another possible benefit would be masking of parasite surface antigens and therefore preventing antibody opsonization and phagocytosis. Indeed, tighter rosettes formed with blood group A RBCs have been shown to mask PfEMP1 from antibody recognition [177]. Furthermore, rosetting has been observed to hamper the phagocytosis of pRBCs [178,179], and rosetting could be a mechanism to reduce the blood flow, which would encourage for endothelial cell adhesion. On the other hand, the surrounding uRBCs can serve as a barrier blocking the pRBC's adhesion to endothelium [180,181]. Alternatively, during high local parasitemias, vascular endothelium might be too saturated for pRBC adherence, and secondary binding to uRBC occurs as endothelium and RBCs share many receptors. Regardless of the exact purpose of this phenomenon, it seems to play an important role in disease pathogenesis. Several host and parasite receptors mediating cytoadherence have been identified and will be reviewed in the following sections.

1.4.3.1 Adhesive parasite ligands

PfEMP1

PfEMP1, which appears on the pRBC surface around the same time mature trophozoites disappear from circulation, is the most noteworthy surface adhesin. In addition, PfEMP1 is a known target for naturally acquired immunity [182–186]. This relatively large protein (200–350kDa) is encoded by the *var* gene family, which undergoes antigenic variation (see section 4.4) [187–189]. Each haploid parasite genome contains approximately 60 variants, generating a bouquet of possible adhesive phenotypes [190]. *Var* genes can be divided based on their upstream elements and the transcription orientation to UpsA, UpsB, UpsC, and UpsE [191–193]. UpsA genes are located in subtelomeres and transcribed toward the telomer end, UpsC

genes are located internally, and group B genes are found in both locations. Some clinical observations have been made about the groups and manifestations of malaria; for example, group A expressing isolates are linked to severe disease in Africa [172,194–196]. The *var* gene encompasses two exons divided by a conserved intron. The first exon encodes for hypervariable extracellular part of PfEMP1, including the N-terminal segment (NTS), the C2 domain, and the multiple adhesive domains of DBL and cysteine-rich interdomain (CIDR) [197]. The second exon encodes a semi-conserved acidic terminal segment (ATS) that includes a C-terminal transmembrane region [190]. The DBL and CIDR domains can further be divided into five (α, β, γ, δ, and ε) and three (α, β, and γ) classes, respectively [197]. The number and order of adhesive domains may vary, creating a wide assortment of proteins. In addition to cytoadhesive properties, binding of PfEMP1 has been implicated in immune modulation [198].

RIFINs

The largest family of variable surface antigens is the repetitive interspersed (RIFIN) protein family encoded by more than 150 *rif* genes per genome [199]. *Rif* genes are composed of two exons and each pRBC only expresses a few *rif* genes at the time [200–203]. The first exon contains a predicted signal peptide, and the second exon encodes the protein itself and contains extracellular conserved cysteine residues with a highly variable region, transmembrane segment, and conserved intracellular tail [200]. Unlike PfEMP1, RIFINs are typically small proteins (20–40 kDa) with one transmembrane domain [204,205]. Based on the presence or absence of a 25 amino acid insertion, RIFINs can be dived into two groups: A-RIFINs and B-RIFINs [199]. This sub-division has functional significance as the A-RIFINs are transported via MC to the surface of the pRBC, whereas B-RIFINs remain confined within the parasite [199–201,206,207]. The function of RIFINs remains elusive, but some light has been shed on their contribution to disease pathology. Like PfEMP1s, RIFINs are clonally expressed and targets of protective antibodies [208,209]. Goel et al. linked an A-RIFIN (PFIT_bin05750) to the formation of rosettes in blood group A [204]. Recently, RIFINs have gathered attention for their immunomodulatory function by binding leukocyte receptors [210,211].

Other surface exposed parasite antigens

The Sub-Telomeric Variable Open Reading Frame (STEVOR) protein family is closely related to RIFINs [212]. Like RIFINs, STEVORs are small proteins (20–40 kDa) that are believed to be expressed in a mutually exclusive manner. The function of STEVORs is puzzling. They are found on pRBC surface [213] during the merozoite stage (apical end and surface) [214–216] and expressed in sporozoites and gametocytes [217]. Therefore, it is likely that they perform different tasks in each parasite stage. Niang et al. provided some clues to the STEVOR's function and demonstrated that it bound glycophorin C (GYPC) on RBCs and therefore mediates rosetting [215,218]. Moreover, anti-STEVOR antibodies have been observed to inhibit parasite invasion in a variant-specific manner [218]. This targeted inhibition implies that STEVORs could contribute to disease pathology; moreover, antibodies against them have been observed in the sera of malaria-exposed individuals [219].

Another intriguing family of surface exposed proteins is composed of the SURFINs that are encoded by the surface-associated interspersed genes (*surf*). As with the RIFINs and STEVORs, they are expressed at different parasite stages and cellular locations [220]. $SURFIN_{4.2}$ has been implicated in merozoite invasion and found at the knobs with PfEMP1 [221,222]. The fifth multigene family of 13 members encodes for Maurer's clefts two transmembrane (PfMC-2TM). Whether they are exposed or not on the pRBC surface remains inconclusive [205,223–225].

1.4.3.2 Host receptors

Different members of the parasite adhesin families have affinity to different host receptors and several interactions can happen simultaneously. The site of sequestration influences the manifestations of the disease. Some of the more studied host receptors and their associated pathologies are described below.

CD36

The cluster of differentiation 36 (CD36), also known as platelet glycoprotein 4, is a receptor displayed on platelets, monocytes, RBCs, microdermal endothelium, dendritic cells, and some other cell types [226]. CD36 is an important scavenger receptor mediating non-complement dependent phagocytosis of various pathogens including malaria [227,228]. Most of the clinical isolates and laboratory parasite strains bind this receptor through PfEMP1, which leads to rosette formation and endothelial cytoadherence [229–234]. Parasite adherence to CD36 might protect it from phagocytosis mediated by CD36. Despite the promiscuous binding to CD36, no clear correlation between disease severity and CD36 binding has been observed [229,230]. Rather, the malaria cases with predominantly CD36 adhering parasites were associated with uncomplicated disease [235,236]. Furthermore, a wide abundance of polymorphisms of CD36 gene have been identified in malaria endemic areas. Depending on the study site and population the results differ regarding whether there is protection from severe malaria [237–241]. This might be because of CD36's other functions including immune responses to other diseases which would lead to balanced selection between functionality and reduced parasite sequestration.

Intercellular Adhesion Molecule 1 (ICAM1)

ICAM1 is present in low abundance on endothelial cells and leukocytes but is drastically upregulated upon activation by cytokines or binding of pRBC [242,243]. The parasite ligand that mediates the interaction appears to be PfEMP1 [244]. In other words, the binding to endothelial cells activates the cells and increases the expression of ICAM1, which allows for more pRBC to adhere. This cycle might be behind disease pathology associated with ICAM1 binding. Post-mortem analysis from brains of fatal *P. falciparum* patients revealed significant co-localization of ICAM1 and sequestered pRBCs [234]. Moreover, the binding of ICAM1 was the highest in cerebral malaria isolates [229,235]. In addition, the dual binding of ICAM1 and endothelial protein C receptor (EPCR) has been linked to cerebral malaria pathogenesis and breakdown of the blood brain barrier (BBB) as the pRBC seems to be internalized by brain

endothelial cells in an ICAM-1-dependent manner [245,246]. Although the heterozygous genotype of ICAM1 is associated with lower *P. falciparum* parasite density, no correlation with protection from severe disease have been noted [247,248].

Endothelial protein C receptor (EPCR)

EPCR, a receptor for activated protein C, is found on the surface of endothelial cells in the microvasculature and of larger blood vessels. EPCR is also expressed on other cell types such as placental trophoblasts and neutrophils. Several studies suggest that PfEMP1 and EPCR interaction is involved in cerebral malaria pathogenesis [249–252]. EPCR is essential for maintaining stability of the blood circulation through activation of protein C, which has anti-coagulative and anti-inflammatory properties to protect the vascular barrier. Thus, the pRBC adherence to EPCR promotes a pro-coagulative environment and threatens microvasculature integrity. Therefore, it is not surprising that EPCR-binding isolates are associated with clinical manifestations of severe disease, such as retinopathy, anaemia, and cerebral malaria [236,252–257].

Heparan sulphate (HS)

Heparan sulphate (HS) is abundant on all the cell surfaces in the body, including the RBC membrane [258]. This widely expressed receptor allows alternative binding of pRBCs to endothelial cells and uRBCs. HS belongs to the glycosaminoglycan (GAG) family and is closely related to heparin. Heparin and other sulphated glycans can disrupt rosettes and inhibit cytoadhesion of some isolates [181,259–262]. Yet again, the parasite ligand mediating the binding appears to be PfEMP1 [181,262,263]. As with the previously described receptors, most parasites also bind HS [263,264]. Moreover, parasites isolated from patients with severe malaria more readily bound heparin than the ones from mild malaria patients [264]. The potential use of heparin derivatives as malaria treatment was described above, but interestingly, this interaction has also inspired attempts to develop new delivery methods for antimalarials [265,266].

Complement receptor 1 (CR1)

Complement receptor 1 (CR1) is a glycoprotein found at various levels on RBCs, leukocytes, and dendritic cells. This receptor has been proposed multiple roles in malaria pathogenesis. First, CR1 is linked to parasite rosetting via PfEMP1, as CR1-deficient RBCs do not form rosettes and antibodies against CR1 and soluble CR1 can reverse rosetting [267,268]. Second, CR1 is the receptor used to eliminate complement-decorated pRBCs [269]. Third, it is an invasion ligand for merozoites [270]. Population studies on polymorphism have resulted in contradicting findings depending on the study location and malaria endemicity [165]. This is illustrated by the Opi et al. study in children in Kenya, where they saw opposing association with cerebral malaria from two polymorphisms widely spread in Africa [271]. The widespread existence of different polymorphisms could be explained by survival advantage of one of the mutations against other infectious diseases [271].

Chondroitin sulphate A (CSA)

The strongest link between disease pathology and cytoadhesion is pregnancy-associated malaria (PAM). Chondroitin sulphate A (CSA) is a GAG found in very high abundance on syncytiotrophoblasts in placenta. Parasite isolates from placenta commonly bind to CSA, whereas other isolates rarely do so [272–274]. *P. falciparum* uses a unique PfEMP1 called VAR2CSA to sequester in this immune privileged environment [193,273,275]. Recently, involvement of another parasite ligand has been suggested – Plasmodium falciparum chondroitin sulphate A ligand (PfCSA-L) [276]. However, further functional studies are needed to confirm the importance of this ligand.

Blood group (Bg) antigens

The histo-blood group ABO system divides the blood based on presence or absence of A- and B- antigens. These antigens, if present, are classically found on RBCs as well as on endothelial blood vessels, in tissue fluids, and some other cells [277]. Bgs are a result of differences in glycosyltransferases attaching oligosaccharides (A/B-antigens) to acceptor molecules and believed to be evolutionarily beneficial for resisting various diseases. Many subgroups of the major four Bgs exist. For example, several subtypes of A-antigen have been described with two of the more common ones being A1 and A2. Quantitative as well as qualitative differences between these subgroups have been characterised [278]. A possible relationship between ABO blood groups and malaria was put forward in 1967 and has inspired numerous studies [279]. As with many other receptors, the findings are not always coherent, but an overall protective effect of BgO against severe malaria has been seen in epidemiological [280,281] and genome-wide association studies [282–284]. These findings are supported by mechanistic studies investigating the effect of the ABO system on primarily rosetting [177,204,281,285] as well as on cytoadherence [204]. Various members of the PfEMP1 and RIFIN families are believed to mediate binding to Bg antigens [204,285,286]. Counterintuitively, preferential invasion of BgO RBCs has been described although for a small sample size and seemingly in a strain-dependent manner [287–289]. ABO locus has also been linked to different levels of soluble serum factors, such as ICAM1, interleukin-6, P-selectin, S-selectin, and von Willebrand factor (vWF) [277,290]. To sum up, the protective or susceptive effects of Bgs can be confounded by other factors, which may affect the analyses of the role of ABO in disease pathogenesis.

Other human receptors

There are many more human receptors that have been suggested to mediate cytoadhesion. In addition to CSA, hyaluronic acid is present on the placental lining and has been identified as a receptor for parasite adhesion [291,292]. Platelet endothelial cell adhesion molecule 1 (PECAM1) expression on endothelial cells is concentrated to intracellular junctions where it appears to serve as a docking point for pRBCs [293,294]. Furthermore, the activation of endothelial cells upregulates the expression of vascular cell adhesion protein molecule 1 (VCAM1), E-selectins, and the release of P-selectins, which all allow for further adherence of pRBCs [233,234,295–298].

Serum factors

Serum proteins can be essential for rosetting as they might form inter-molecular bridges between host receptors and parasite ligands [299]. Early findings reported observations of fibrillar structures on cytoadhesive pRBCs that contained immunoglobulin (Ig) M and possible IgG [300]. Later, studies found that non-immune IgM plays a role in rosetting for both laboratory clones and clinical isolates [299,301,302] and that IgM binding associates with severe malaria [303]. Non-immune IgM binding PfEMP1 proteins are rather common in genomes of laboratory strains [304,305]. IgM seems to be important for clustering of PfEMP1 on pRBC surface, which increases its binding affinity [306]. The role of IgG is less clear. IgG has been found on placental binding pRBCs and hypothesised to connect PfEMP1 with neonatal Fc receptor [307]. In addition, complement factor D together with albumin and anti-band 3 antibodies have been shown to induce *de novo* rosetting, but the exact mechanism is unknown [308]. Serum proteins, fibrinogen, and vWF, known to participate in rouleax formation, have also been implemented in serum-dependent rosette formation for some parasite strains [302]. Moreover, serum factor dependent type two rosetting was described in *P. falciparum* and *P. vivax* isolates [178]. This rosetting requires monocyte secreted insulin growth factor binding protein 7 and two serum proteins – vWF and thrombospondin-1.

1.4.4 Antigenic variation

For the parasites, there is a grave risk of expressing proteins on the surface of pRBCs as they mark the cells for the immune system, in particular if they are to be exposed for an extended time. Therefore, it is crucial for any pathogen to change surface proteins to prevent elimination. Antigenic variation is a common strategy among pathogens establishing chronic infections, such as *Trypanosoma brucei* [309], *Babesia bovis* [310] and *Giardia lamblia* [311]. Naturally, longer infections increase the chances of transmission to new hosts. Moreover, antigenic variation allows the pathogens to re-infect already exposed hosts. In addition, the wide array of possible parasite ligands creates a spectrum of phenotypes for invasion, cytoadhesion, and solute transporters. Hence, different pathways can be used in the absence of a specific host receptor. However, to avoid premature exposure of all antigen variants, strict control over gene expression is required.

Genes encoding proteins with different roles in parasite biology have been identified as antigenically variable. The best-known example is *var*/PfEMP1 family. *Var* genes are expressed in a mutually exclusive fashion with one or very few dominant *var* genes being transcribed out of ~60 family members [312–316]. Typically, a single PfEMP1 variant or occasionally two are detected on the surface of pRBCs and determine the adhesive properties of the parasite [312,315,316]. To exhaust the repertoire too quickly, an intrinsic low switch rate exists, that is controlled at the transcription level [313,317]. Although not fully understood, the switching seems to be non-random and follows a relaxed hierarchy, at least *in vitro* [318–320].

The smaller surface antigens, RIFINs and STEVORs, are also believed to be variantly expressed within clonal parasite lines [201,321]. This expression does not appear to be

mutually exclusive as multiple *rif* and *stevor* genes can be transcribed simultaneously [207,218,322].

Presumably, the most vulnerable stages of *Plasmodium* life cycle are the ones directly exposed to the bloodstream, such as merozoites invading new host cells. The invasion process involves numerous parasite proteins and adhesive ligands mediating the interaction with the receptors on the RBC. As expected, the invasion ligands are also antigenically variable. Host cell tropism is mediated by members of two multigene families – EBL and Rh (see section 4.1.2). Different specific pairings of parasite ligands and host receptors allow for the use of alternative invasion pathways. The best example for this is an inducible switch from sialic acid-dependent invasion of parasite ligand EBA-175 (member of the EBL family) to sialic acid-independent invasion via Rh4 (member of Rh family) by the removal of sialic acid by neuraminidase [270,323,324].

Variability of nutrient acquisition could help the parasites adapt to different nutritional environments in human host or to survive drug pressure. For example, variant expression has been observed for gene families involved in lipid metabolism such as acyl-CoA synthases [321,325]. In addition, mutually exclusive expression has been noted for *clag3.1* and *clag 3.2* encoding the CLAG3 protein, which is part of the PSAC nutrient channel [117,321]. Interestingly, switching in between these has been associated to blasticidin S resistance [118,326].

1.4.4.1 Regulation of antigenic variation

Antigenic variation is regulated on several levels, but mainly transcriptionally. DNA accessibility for transcription has been suggested to act as the centre piece in coordinated gene regulation [327,328]. First, physical organisation of chromatin in the nucleus has been indicated as a regulatory mechanism linked to DNA accessibility. Most chromosomal regions are in decondensed euchromatin, which allows transcription, but silence genes are associated with heterochromatin. The telomeric regions of chromosomes are kept in repressive heterochromatin in the nuclear periphery, of which four to seven clustered nuclear foci are formed [329]. The active *var* gene, although located in nuclear periphery, is excluded from these clusters, suggesting that the active *var* gene moves to a more euchromatic region where transcription is permitted [330]. Similar relocation before activation has been observed for *PfRh4* locus [331].

The chromatin organization at the local level appears equally important as the global compression of chromatin. The chromatin structure depends on two processes: post-translational modifications of histone tails and occupancy of nucleosomes, which includes binding frequency, strength, location, and protein composition of the nucleosomes. Histone modifications also influence the overall compaction of chromatin. Loci containing clonally variant multigene families are enriched in the repressive histone mark H3K9me3 and heterochromatin protein 1 [331–335]. H3K4me3 and H3K9ac correlate with active *var* genes, and the variant poised for expression in the next cycle is marked by H3K4me2-3 [336]. Direct

DNA modifications, such as methylations of cytosines and adenines, are present at low levels in the *Plasmodium* genome and its gene regulatory role, if any, is not known [337–339].

In addition to epigenetic regulation, participation of non-coding RNAs (ncRNAs) and specific transcription factors have been noted (reviewed in [340]). The introns of *var* genes contain a bidirectional promoter from which sense and anti-sense ncRNA are produced [341]. The expression of anti-sense long ncRNAs are associated with active *var* gene [342]. The exact role of sense and antisense ncRNAs is unknown, but both have been observed incorporated into the chromatin [342,343]. Additionally, a family of GC-rich ncRNAs has been demonstrated to act on *var* and *Pfmc-2TM* gene regulation [344–346]. The role of ncRNAs have also been implicated in the regulation of *clag* genes [115]. For transcriptional control of *rifins* and *stevors*, AP2-exp, a member of Apetala2 (AP2) family of transcription factors, could be important [347]. Lastly, peculiar translational regulation has been shown for one PfEMP1, VAR2CSA, which needs a particular translation factor for the production of a functional protein [348,349].

1.5 PATHOGENESIS

1.5.1 General malaria manifestations

As symptoms of uncomplicated malaria are very generic such as fever, malaise, fatigue, headaches, nausea, and chills, they can be easily confused for any other common infection [350]. The appearance of symptoms is correlated to the emergence of the parasite in the bloodstream and the beginning of IDC (8–15 days post infection). Most of the manifestations can be explained by the release of toxic compounds, such as hemozoin and glycosylphosphatidylinositols, into the bloodstream when the merozoites egress and the cell lyses [351]. These toxic products stimulate macrophages and other cells to secrete cytokines and other factors that will induce rigors, fever, and possibly other symptoms [351–353]. Synchronous schizogony of parasites results in the onset of fever paroxysms characteristic of malaria. However, febrile episodes can also be irregular as sometimes observed for *P. falciparum* and *P. knowlesi* infections. The timing of fever peaks depends on the *Plasmodium* species and their corresponding IDC completion time. In addition, repeated malaria infections are often accompanied by splenomegaly. If not treated, malaria can rapidly progress to severe illness and death.

1.5.2 Severe malaria

The main cause of severe malaria is *P. falciparum*, but it is not the only culprit as severe disease and fatal outcome can also follow an infection with *P. vivax* or *P. knowlesi*. Complications occur when the patient experiences serious organ failures and/or abnormalities in the metabolism or blood. These complications require urgent treatment and qualify severe malaria as a medical emergency. Epidemiological definition of severe malaria includes one or more of the following: impaired consciousness (cerebral malaria), acidosis, hypoglycaemia, severe malarial anaemia, acute kidney injury, jaundice, pulmonary oedema, abnormal bleeding, shock, and hyperparasitemia [4]. Syndromes of severe malaria often overlap [4,354]. The case fatality rate of severe malaria varies substantially among countries depending on access to in-patient

care; however, even if treated, it is around 20% [4,355]. Although the pathogenesis of severe malaria manifestations is not fully understood, some of the possible causes are described in following sections.

1.5.2.1 Severe malarial anaemia

As anaemia is common in malaria endemic areas, severe malarial anaemia is classified only in the presence of parasites (>10 000/μL) in combination with lower than normal haemoglobin (< 5g/dL in children and < 7g/dL in adults) and haematocrit (< 15% in children and < 20% in adults) [4]. Depletion of oxygen-carrying haemoglobin/RBCs in anaemia results in reduced oxygen carrying capacity of blood leading to asphyxia. The mechanisms underlying the anaemia are multiple with the obvious first suspect being the sequential invasion and rupture of RBCs by the parasites. Another predictable reason is opsonization and clearance of pRBCs by the immune system and splenic clearance of rigid pRBCs. However, this does not account for the entire loss of RBCs. In fact, the main reason for the rapid decline of haematocrit is believed to be due to lysis of uRBCs. Mathematical modelling suggests that an average of 8.5 uRBCs per pRBC are destroyed [356]. This could be the result of failed invasions during which merozoites have deposited parasite-derived proteins onto the surface of uRBCs, such as rhoptry-derived ring surface protein-2 (RSP-2), and marking them for destruction [357,358]. To make matters worse, malaria infection suppresses erythropoiesis. RSP-2 has also been observed to tag erythroid precursor cells in the bone marrow possibly contributing to dyserythropoiesis [358]. Moreover, erythropoiesis can directly be suppressed by released hemozoin during the egress and additionally by inflammatory mediators – tumour necrosis factor-α (TNF-α) and nitric oxide (NO) – produced by hemozoin-activated immune cells [359–361]. Malarial infection also causes other abnormalities in blood including leukopenia, thrombocytopenia, and hypoglycaemia [362].

1.5.2.2 Cerebral malaria

Cerebral malaria (CM) is diagnosed in malaria patients with unarousable coma for more than one hour after convulsions (not explained by other causes) using coma scales: in a Blantyre coma score of less than 3 of 5 or in adults a Glasgow score of less than 11 of 15 [4]. This neurological manifestation is the most severe malaria manifestation with 15–20% fatality rate even when treated, and 100% fatal without treatment [363]. Moreover, many who recover suffer from neurocognitive sequelae including ataxia, paralysis on one side of the body, speech disorders, and blindness. CM presents differently in adults than in children. Despite the lower mortality in paediatric CM, children are more prone to seizures and post-CM neurocognitive deficits [364,365]. The pathogenesis of CM could be explained by two approaches (mechanical sequestration based or cytokine centred view), but pathogenesis is probably due to their combination. Sequestration of parasites in the brain microvasculature of CM patients is commonly noted in post-mortem examination [366,367]. This sequestration has been proposed to advance the pathology in various manners, including disruption of blood perfusion, inflammation, and endothelial dysfunction. For example, pRBC binding of EPCR on endothelial cells via PfEMP1 has been linked to CM and brain swelling [252,254,368].

Microvascular congestion might lead to local hypoxia at the obstruction site and increase intracranial pressure in neighbouring areas. Additionally, imbalances of pro- and anti-inflammatory cytokines have been implicated in CM [369]. Increased levels of cytokines (pro- and anti-inflammatory) were found in Malawian children suffering from acute CM [370]. Several studies in humans have implicated elevated TNF-α in CM [371]. Ultimately, the activation of endothelial cells in combination with parasite factors leads to the loss of BBB integrity. Parasite expressing PfEMP1-s with dual-affinity to EPCR and ICAM-1 have been shown to induce the uptake of pRBCs by brain endothelial cells and cause the breakdown of BBB [245]. Moreover, examination of CM brain tissues showed reduction in junction proteins co-localized with sequestered pRBCs [372,373].

1.5.2.3 Respiratory distress

Respiratory distress, which is characterized by deep and laboured breathing, is prevalent among malaria patients and an important sign of severe malaria. Malaria-associated acute respiratory distress syndrome (MA-ARDS) is a serious complication with high mortality rate. MA-ARDS pathogenesis is associated with damaged alveolar-capillary membranes and alveolar inflammation, which results in alveolar oedema and ultimately hypoxemia. In many instances, pathology occurs after treatment with antimalarials [374]. As with the other outcomes, MA-ARDS is likely multifactorial. Parasite hemozoin has been suggested to be one of the culprits as it accumulates in lungs of rodents and humans where it stimulates the lung epithelium [375–377]. Various models show that the epithelial activation leads to upregulation of adhesion ligands (CD36 and ICAM1) and proinflammatory mediators [376,377]. Moreover, in a mural ARDS model, accumulation of pRBCs resulted in cross-presentation of parasite antigens by endothelial cells to $CD8^+$ T-cells, which drives the breakdown of tight junctions and damage to the alveolar-capillary membrane [378]. Sequestration of pRBCs in lung microvasculature has been observed in malaria patients [366,379]; in a murine malaria model, sequestration via CD36 is linked to acute lung injury [380]. Moreover, post-mortem lung sections of MA-ARDS patients showed increased intravascular vWF, alveolar oedematous vWF, and angiopoieting-2 levels, findings that suggest endothelial activation [381].

Typically, metabolic acidosis manifests clinically as respiratory distress, namely hyperventilation. Metabolic acidosis is defined as excessive acidity of the blood and tissue fluids. In response, the body attempts to overcome the decrease in pH by altered breathing pattern to expel more carbon dioxide. Metabolic acidosis has been found to be a major risk factor for fatal outcome [382–385]. Acidosis can develop due to hepatic dysfunction, renal impairment, and/or microvascular obstruction [4]. Furthermore, a major contributor is anaerobic glycolysis in hypoxic cells due to parasite vascular occlusion and anaemia [386]. In addition, parasites consumption of glucose is intimately linked to the parasites' secretion of lactic acid and free protons as waste, which contributes to the development of acidosis [387].

1.5.2.4 Pregnancy associated malaria

In endemic areas, children never develop sterile immunity to the parasite but acquire immunity to severe disease through repeated exposure to the parasites. However, pregnancy renders semi-immune women again susceptible to severe disease, which harms both the mother and the unborn child. Any manifestation of severe malaria may occur in the mother, but respiratory distress and hypoglycaemia are the most common. PAM leads to an estimated 10 000 maternal and 200 000 neonatal deaths a year [388]. Even the surviving foetuses suffer severe consequences such as growth retardation. The placenta presents a new growth niche for the parasites and by adherence to the CSA on syncytiotrophoblasts, excessive sequestration of pRBCs to placenta is commonly observed [275]. In addition to a heavy parasite biomass blocking the nutrient and blood flow to the foetus, inflammatory responses to the sequestered parasites are believed to cause substantial harm. An infiltration of activated immune cells to the placenta and dysregulation of soluble mediators are associated with placental pathologies [389–392]. The primigravidae are at the highest risk of developing PAM, but the risk drops considerably with successive pregnancies [393]. Sera from multigravida women can block adhesion to CSA [394,395]. Furthermore, antibody levels and the ability of the antibodies to block the binding to CSA are parity-dependent [396] and improve the clinical outcome of PAM [397–400].

1.5.3 Determining factors of severe disease

A wide range of parasite and host factors dictate the disease outcome such as parasite load, sequestration preference, host immune status, and balance between pro- and anti-inflammatory responses. There are differences in disease manifestations between adults and children, low vs. high transmission areas, and even due to gender. In high transmission areas, severe malaria occurs more often in children under the age of five with severe anaemia being the most common complication; however, in lower transmission areas, slightly older children are threatened by cerebral malaria [4]. Children progressively acquire immune protection through continuous exposure to the parasites – first against development of severe disease, then to uncomplicated malaria, and finally to any clinical manifestations. Unfortunately, sterile immunity seems to be hard to achieve (if ever), and the acquired immunity is lost quickly when exposure is eliminated. Therefore, it is not surprising that in lower transmission areas, severe disease can occur in any age groups as the development of immunity takes longer [401]. Even when clinical immunity is achieved, women are rendered susceptible to severe disease when they become pregnant as discussed above.

Susceptibility to severe disease may be influenced by other ongoing infections in the host. Not only can these infections make correct diagnosis more difficult but also worsen the pathogenesis of malaria. The most studied is the co-infection with human immunodeficiency virus (HIV), which carries a higher risk of infection, elevated parasite numbers, more complications, and higher fatality rate [402–404]. HIV seropositive pregnant women are more likely to transmit the virus to the foetus when infected with malaria [4,405]. Furthermore, HIV status seems to hamper the antimalarial immunity development [406–408].

Many inherent RBC disorders are common in malaria endemic areas and are strongly linked to protection from severe malaria. Evolutionary balance between clinical disease and harm from malaria has been achieved in endemic regions. The best-known disorder is probably sickle cell disease, where homozygous individuals for the sickle cell gene suffer disease pathology but heterozygous carriers do not have the haemoglobinopathy and benefit from the protection from severe malaria thanks to the reduced parasite growth at low oxygen tension. Several other protective mechanisms have been discovered. Moreover, hetero- and homozygotes for haemoglobin (Hb) variants C and sickle cell carriers have reduced parasite cytoadherence. In addition, hereditary elliptocytosis, where RBCs are elliptical, hampers parasite invasion. Both glucose-6-phosphate dehydrogenase (G6PD) deficiency and HbAE reduce parasite densities [4]. The underlying mechanisms of some of those hemoglobinopathies are known. In HbAS and HbS pRBCs, the trafficking of PfEMP1s and other proteins to the cell surface is perturbed, resulting in reduced cytoadherence [162,409]. Genetic diversity in other genes encoding RBC and endothelial receptors also occurs, but the connection to protection from malaria is not always clear.

2 RESEARCH AIMS

This thesis aims to further the understanding of malaria disease dynamics by investigating the features of *P. falciparum* related virulence and fitness. The role of surface antigens and cytoadhesion was a red thread throughout the studies. Specific aims of each paper are listed below.

Paper I: To elucidate the rosetting characteristics of *P. falciparum* in the ABO histo-blood groups with emphasis on weak ABO subgroups.

Paper II: To assess and authenticate reagents for investigation of the RIFIN family proteins implicated in rosetting.

Paper III: To characterize the effect of blood pH on surface antigen expression.

Paper IV: To investigate the mechanisms behind heparinoid invasion inhibition and how heparinoids affect the intracellular growth of *P. falciparum* and consequently its virulence features.

3 METHODOLOGICAL CONSIDERATIONS

This section presents the methodological considerations of laboratory methods of particular importance. Detailed protocols are available in the *Materials and methods* section of the respective papers.

Parasite cultivation

P. falciparum laboratory strains and clinical isolates were cultivated following standard methods [410] with minor modifications to meet the needs of the experiments. In general, parasites were grown in RPMI-1640 supplemented with 10% A+ human serum in BgO erythrocytes under constant microaerophilic condition. In **paper I**, experiments involving erythrocytes of various Bgs, 10% AB+ serum for laboratory strains and 15% AB+ serum for clinical isolates were used to avoid incompatibility between serum and erythrocytes. To investigate serum effect on rosetting (**paper I**), 10% of serum from each donor was used with pooled O+ erythrocytes. In **paper III**, pH of culture medium was adjusted using hydrochloric acid and sodium hydroxide with no changes to blood components. Previous studies have shown that PSAC inhibition depends on glucose availability [113]. Hence, in **paper IV**, we aimed to investigate sevuparin's/heparin's effect on parasite intracellular development under more physiological glycose levels. As the normal RPMI-1640 medium contains a high nutrient concentration, we reduced the glucose concentration in culture medium from 2 g/L to 1.2 g/L (normal blood glucose level is 0.7-1.3 g/L).

Selection of parasite phenotypes

Rosetting phenotypes of parasites were maintained by enrichment over a Ficoll-gradient, where heavier rosettes are separated from uRBCs and non-rosetting pRBCs [411]. Binding of CSA was achieved by selection on CSA-coated plastic plates as described by Brolin et al. [412]. In both cases, several rounds of selections were necessary to produce strong phenotypes. Furthermore, as a slow drift in dominant *var* gene expression occurs, regular enrichments were required for maintenance.

Rosetting capacity was evaluated regularly by counting the percentage of rosette forming mature trophozoites under light microscopy. Phenotypes were further confirmed by immunostaining against dominant PfEMP1 and flow cytometry as discussed below. For the VAR2CSA expressing parasites, capacity to bind placenta was evaluated using an assay adapted from Flick et al. and Rasti et al. [292,307]. Magnetically-enriched pRBCs were allowed to bind placental cryo-sections for 1 h during incubation at 37 °C in humid chamber. Thereafter, the unbound cells were washed away and the binding quantified by microscopy. We observed that the number of bound parasites depended on various factors, including hematocrit, placental section, and relative humidity. Therefore, a non-VAR2CSA expressing parasite line (NF54CSA-ptefKO [348]) was used as internal control for normalization.

Moreover, dominant *var* gene expression was confirmed using RT-qPCR and gene specific primers. RNA from ring stage parasites (8–16 hpi) was collected and reverse transcribed. cDNA was probed with dominant *var* gene specific primers and with primers for endogenous control (*seryl-tRNA-synthetase*).

Flow cytometry

Flow cytometry was one of the key methods employed in the papers due to its objectivity and high throughput (Fig. 4). All assays were performed using FACS-Verse with universal loader (BD Bioscience) and analysed with FlowJo software (BD Bioscience).

To determine parasitemia, parasite stage distribution, and rosetting rate, Hoechst 33342 and Dihydroethidium (DHE) co-staining was used [413,414]. As uRBCs do not contain any DNA, any cell positive for Hoechst would be parasitized giving the total parasitemia. DHE is oxidized in cells, where it stains both DNA and RNA, which allows for the determination of "late stage" parasites (mature trophozoites and schizonts). Rosetting characteristics could be evaluated from the "late stage" parasite population. Single cells are commonly used for flow cytometric analysis as determined by equal size of events on forward scatter (FSC) – height vs. FSC-area blot. Ch'ng et al. showed that events that have a larger area than height, so called mutliplets, can be used as surrogates for rosetting rate [414]. In **paper I**, this method was developed further by lowering FSC and side scatter (SSC) voltages and by eliminating the primary FSC-SSC gating conventionally used for debris removal. This improved the correlation between rosetting rate observed by microscopy and the percentage of multiplets. Increased sensitivity meant that small changes in rosetting rate between different conditions could be quantified with high confidence. In addition, the SSC area was observed to be a good indication for relative rosette size and could be used to compare the rosette sizes between conditions. In **paper IV**, we observed that extracellular parasites can be quantified from the same Hoechst-DHE staining by gating for Hoechst positive cells with relatively low FSC area. This gating was confirmed by releasing parasites from pRBCs using saponin lysis.

For the detection of PfEMP1 surface exposure/accessibility on the pRBCs, cells were incubated with primary goat antibodies raised against dominant PfEMP1 and then incubated with anti-goat secondary antibodies conjugated to a fluorophore. Non-immune goat IgG was used as a control to establish the level of non-specific binding. Non-immune IgG binding has been reported for placental binding parasites [300,415], although binding of IgM is more prevalent [299,416,417]. The same principle was used in **paper I** to determine A/B-antigen levels on RBCs using mouse antibodies as described in Hult and Olsson [418]. In **papers I** and **III**, a combination of Hoechst and Alexa Fluor 488 (AF488) coupled secondary antibodies were used. In **paper IV**, the DHE staining was added (in addition to Hoechst) and therefore Alexa Fluor 647 coupled secondary antibodies were chosen to avoid spill overs. Data were quantified as percentage of PfEMP1 positive "late stage" parasites or as median fluorescence intensity (MFI) of the late stage parasite population.

In **paper IV**, we established a flow cytometry protocol to measure cytosolic cation levels in pRBCs. We tested various indicator dyes that would emit a fluorescence signal upon binding to cations: ION NaTRIUM Green-2Am (Abcam), ION Potassium Green-2Am (Abcam), Fluo4 (Invitrogen), and CoroNa Green AM (Invitrogen). The only dye that produced a strong and sensitive staining was sodium indicator ION NaTRIUM Green at 10 µM (Adcam) with 10 µg/mL Hoechst 33342 (Invitrogen) and 0.01% Pluronic F-127 (Invitrogen) in RPMI-1640 for 1 h at 37 °C. During the analysis, the rosette disruptive properties of the drugs (sevuparin/heparin) had to be considered as the fluorescence intensity of single cells would be much lower than in a rosetting event, creating a bias in the analysis. Therefore, normalized MFIs were calculated as a ratio between the MFI from Hoechst 33342 and ion-indicator double-stained sample and MFI from Hoechst 33342 single stained sample as described for embryonic stem cells [419].

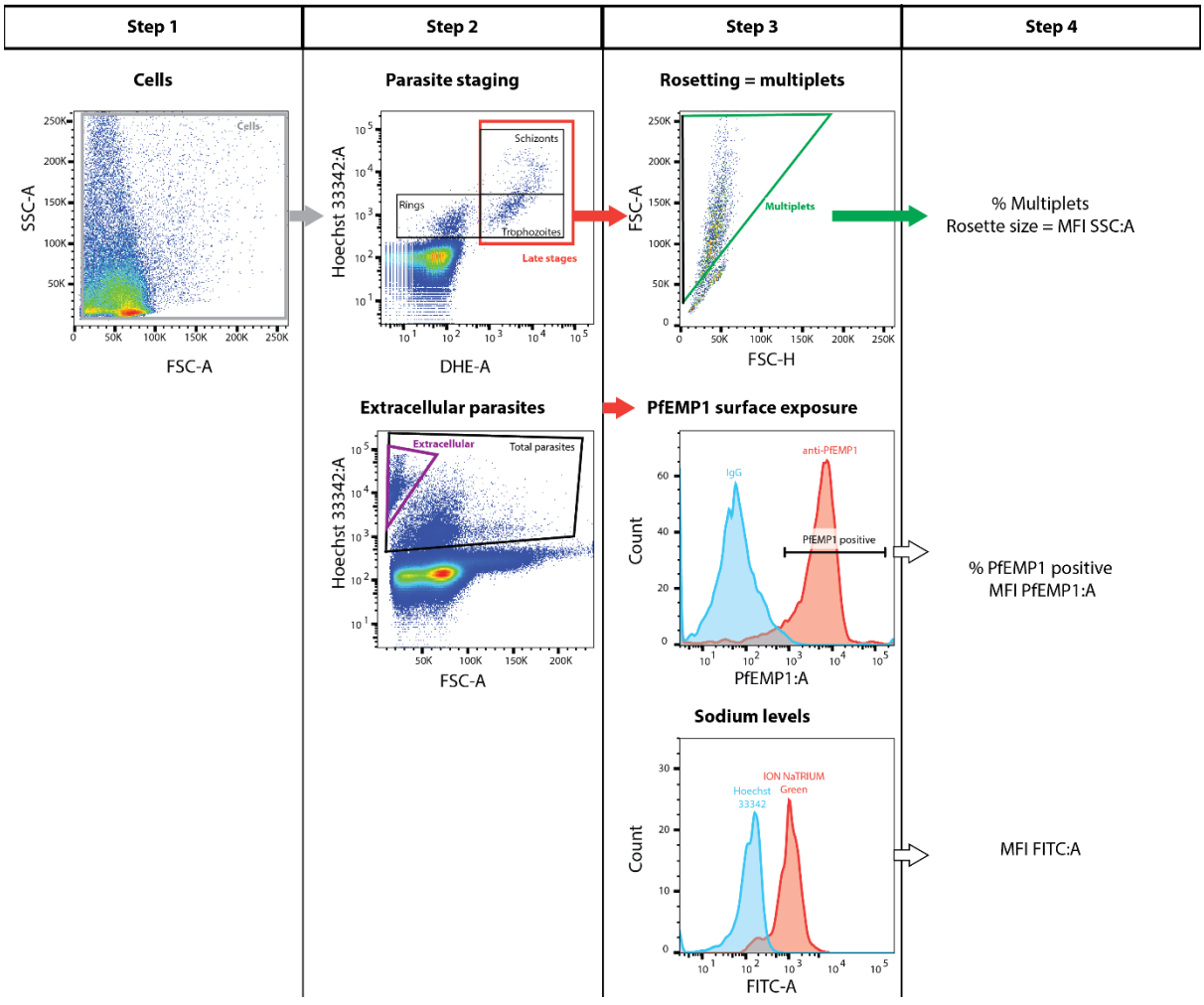

Figure 4 Overview of flow cytometry gating strategies. The first gating is used to exclude debris and choose the cells of interest based on size (grey). In the case of *in vitro* culture, all cells are of interest and therefore this step could be omitted. Thereafter parasite stage, parasitemia and number of extracellular parasites can be obtained. Trophozoites and schizonts constitute a "late stage" population (red) that is used for further analysis: the percentage of multiplets (rosetting rate (green)) and PfEMP1 surface exposure. The total parasite population is used for evaluation of sodium levels using indicator dye (Ion NaTRIUm Green). Additional statistics can be obtained for comparison between samples.

Immunofluorescence assay (IFA)

Visual examination can give additional information about protein cellular location, potential protein interaction partners, and the specificity of the antibody. In this work, many versions of immunostainings were used. In **paper II**, IFA was used to visualize the cellular location of the proteins recognized by the antibody and to investigate the strain-transcending capacity of antibodies. pRBCs were allowed to adhere to poly-L-lysine treated microscopy slides and fixed by desiccation. This procedure breaks the pRBC membrane and allows the antibodies to access the inner surface. However, native confirmations of epitopes might be lost. After blocking with bovine serum albumin, primary antibodies were allowed to adhere for 1 h at room temperature. After washing, corresponding secondary AF488 conjugated antibody was added. Finally, parasites were counterstained with nuclear dye (DAPI or Hoechst 33342) and mounted for visualization. In **paper IV**, the same procedure was used for late schizont pRBCs; however, before addition of primary antibodies, cells were incubated with increasing concentrations (3–250 μg/mL) of sevuparin or heparin for 1 h at room temperature. The results from this competition assay between antibodies and sevuparin/heparin supported findings from other methods regarding heparin binding partners. In addition, live merozoite staining was performed in **paper IV**, which allowed for a more natural interaction. Merozoites were allowed to egress in presence of FITC-labelled heparin or sevuparin. Thereafter, merozoites were collected and fixed with 1% paraformaldehyde. Untreated parasites were stained with anti-RON3 antibodies and then with species-specific secondary antibody coupled to AF594. Both samples were counter-stained with Hoechst 33342. All experiments were visualized with Nikon Eclipse 80i fluorescence microscope.

Lysis assay

Sorbitol lysis is routinely used for parasite synchronization as it depends on presence of PSAC on pRBCs [420]. Similarly, protection from sorbitol lysis can be employed for investigation of PSAC inhibitors. By modifying method from Wagner et al., we probed the possible inhibition of PSAC by sevuparin in **paper IV** [421]. Magnetically-enriched pRBCs were incubated with three concentrations of sevuparin or with known PSAC inhibitor furosemide for 1 h at 37 °C. Phosphate-buffered saline (PBS) was used as a control. Next, samples were placed on ice until analysis. Lysis was initiated by adding the pre-warmed lysis solution (280 mM sorbitol, 20 mM Na-HEPES, 0.1 mg/mL BSA, pH 7.4 with drugs). Cell lysis was monitored at optical density (OD) of 700 nm with Nanodrop 2000c at 37 °C in a kinetic mode with continuous magnetic stirring. As the change in optical density depends on the efficiency of magnetic enrichment of pRBCs during the particular experiment, the results were normalized to the control sample (no drugs added): 100% cell lysis corresponded to the OD change of the control sample at the end of the experiment. All other time-points were calculated as: cell lysis (%) = OD_{start}(drug)-OD_{time}(drug))/(OD_{start}(control)-OD_{end}(control).

Preparation of proteomics samples

Mass spectrometry is a powerful tool to obtain new leads and to support findings from other methods. Here the preparation of proteins from various sample types is described. In **paper**

III, mass spectrometry for identification of peptides shaved from pRBC surface was performed. To determine the differences in exposed proteins on pRBC surface after growth in acidic or normal media, cells were treated with 0, 10, or 100 µg/mL of trypsin (Sigma) for 20 min at 37 ^{0}C. The reaction was stopped with the addition of soybean trypsin inhibitor (Sigma) at 1 mg/mL for 5 min at room temperature. The efficiency of trypsin cleavage was confirmed by flow cytometry and antibodies against dominant PfEMP1, which showed complete loss of staining after the treatment. The cell supernatant containing tryptic peptides was collected and separated on SDS-PAGE. The sample lanes were excised and subjected to nanoLC-MS/MS.

In **paper IV**, mass spectrometry was used to identify proteins interacting with heparin/sevuparin. Purified merozoites were lysed in RIPA buffer on ice for 15 min. Next, potential detergents were removed from the sample with a HiPPR Detergent Removal Resin Kit (Thermo Scientific). Protein lysates were pre-cleared with uncoupled Sepharose beads (GE Healthcare) and then incubated with heparin-coupled or sevuparin-coupled sepharose for 3 h at 4 °C. Beads were washed three times and bound proteins were eluted with SDS buffer. Eluted proteins were separated by SDS-PAGE gel. Gel lanes were excised and analysed by nanoLC-MS/MS mass spectrometry.

For the investigation of intracellular interactors, the empty membranes of pRBC and uRBC known as ghosts were generated by the addition of 5mM KH_2PO_4 (pH 7.4) for 10 min at room temperature. Ghosts were lysed in RIPA buffer and sonicated for 10 min for total membrane rupture. The potential remaining detergents were removed from the sample with a HiPPR Detergent Removal Resin Kit (Thermo Scientific). The incubation with sepharose beads was carried out using the same procedure as with merozoite proteins. Uncoupled sepharose beads were included as controls due to the increased complexity of samples. After the washes, the sepharose beads were resuspended in 100 mM ammonium bicarbonate for analysis by nanoLC-MS/MS.

Peptide array

To determine the specificity of antibodies and map their epitopes, a peptide array analysis was performed in **paper II**. For this purpose, custom made ultra-dense peptide microarray was created in collaboration with Roche-Nimblegen. The array was designed with representative members of multigene families (PHISTs, RIFINs, STEVORs, PfEMP1s, SURFINs, and 2TMs) and contained 175 000 peptides of 12 amino acids in length. One amino acid lateral shift between adjacent peptides was designed to improve differentiation between epitopes and background. At least two adjacent peptides needed to be reactive to constitute an epitope, and any peptide without adjacent reactive peptide was discarded as false-positive.

Ethical considerations

The collection and use of blood products for parasite culture was approved by the Regional Ethical Review Board in Stockholm (Dnr: 2009/668-31/3). Erythrocytes and plasma were obtained through Karolinska University Hospital Blood Bank from healthy Swedish donors. The collection of clinical isolates from Uganda used in **paper I** was approved by Karolinska

Institute's Regional Ethical Review Board (permission 03/095) and the Uganda National Council for Science and Technology (permission MV717). Informed written consent was acquired from the parents or guardians of the patients.

Healthy Swedish placentas used in **paper III** were collected with approval from Regional Ethical Review Board in Stockholm (Dnr: 04-533/2). Approval for the use of human sera from individuals living in endemic regions was obtained from The Committee for Laboratory Safety and Ethics, China (permission 2008-IZ-20). Written informed consent was obtained from the patients.

In **paper IV**, data for IC50 studies of clinical isolates were extracted from previously published work [21] and from experiments with isolates received from European Malaria Reagent Repository and National Institutes of Health (USA).

4 RESULTS AND DISCUSSION

PAPER I

"Low blood group A-antigen levels on red blood cells render *P. falciparum* rosettes more susceptible to disruption".

Several genome wide association and epidemiological studies have linked ABO Bgs to malaria disease severity, notably protection from severe malaria by BgO compared to non-O Bgs [281,282,422,423]. Rosetting via Bg antigens have been suggested to drive the differences in malaria pathology between Bgs. Augmented rosetting rate and rosette size have been observed in BgA compared to BgO [204,281,285]. BgA further divides into subgroups with the two major ones being A_1 and A_2. There are qualitative and quantitative differences between them with A1 expressing approximately five times more A-antigen on the surface of RBCs [278].

In this study, we wanted to characterize the rosetting features of *P. falciparum* in a systematic way to explain some of the increased risk seen in association with Bgs. We were particularly interested in elucidating the role of weak Bg subgroups, which could affect the interpretation of previous results. As increased prevalence of BgO in Africa has previously been suggested to be selected for by malaria [424], we wanted to investigate the relative frequencies of Bg alleles leading to various weak phenotypes. To obtain the allele frequencies from various geographical regions, we took advantage of data available at the Erythrogene database. In accordance with epidemiological studies [424], BgA allele frequencies were the lowest in African cohort, which also suffers from the highest *P. falciparum* malaria burden. Furthermore, the allele frequency for weak BgA alleles was the highest among this cohort (predominantly A2).

To determine the rosetting characteristics from sufficient number of samples and parasite lines, a high-throughput robust flow cytometric method was implemented. The method developed by Ch'ng et al. was able to detect multiplets relative to parasite rosetting rate but failed to determine absolute rosetting rates [414]. To identify subtler changes in rosetting rate expected between blood subgroups, the FCS and SSC voltages of the cytometer were lowered, and the primary gating on FCS-SSC was skipped to include all the rosetting events. Furthermore, SSC-A was used as a surrogate for determination of rosette size. Our updated method was able to measure rosetting rate and size with good correlation to the ones observed by microscopy.

Erythrocyte samples from seven healthy Swedish donors per blood group were collected (in total 28 samples). Two laboratory parasite clones and four culture-adapted clinical isolates (two severe and two uncomplicated) were grown in donor blood and their rosetting characteristics described. The influence of A- and B-antigen on rosetting was strain-dependent and became more evident in the presence of rosette-disruptive heparin. Antibody mediated destruction of pRBC is an important part of host immune defence. Hence the antibody accessibility to PfEMP1 in various blood groups was tested. Decrease in antibody staining was seen for all non-O Bgs for both tested parasite clones.

For the more detailed analysis of A-antigen levels on rosetting, A-preferring laboratory clone FCR3S1.2 was used. The blood from 30 donors per blood group (O, A1, non-A1, B, and AB) was obtained and quantified for blood antigen levels. Higher levels of A-antigen protected rosettes from heparin and antibody mediated rosette disruption with most durable rosettes in Bg A1 then Bg A2 and AB. Two clusters within AB Bg were observed, probably corresponding to A1B and A2B. In addition, we investigated the serum contribution to the rosetting as ABO Bgs also affect levels of serum components. FCR3S1.2 parasites were cultivated in BgO RBCs with 10% serum from 80 different donors (20 per Bg) and rosetting evaluated by flow cytometry. No differences in rosette size or rate and no correlation with A-antigen levels were observed.

This paper offers insights to relationship between ABO Bgs and rosetting. The allele frequency investigation suggested that evolutionary pressure for low expressing BgA could be present in regions where *P. falciparum* is highly prevalent. As microscopic quantification is prone to human error and bias and that the through-put is low, the key to the further systematic work was the optimization of the flow cytometric methodology. With this improved method, we were able to characterize rosetting for several parasite lines in various Bgs. The results were strain-dependent with no clear overall preference for rosetting. These results are to be expected as rosetting depends on other ligands than just Bg antigens. Moreover, the small differences seen could be far more important under physiological flow conditions. As PfEMP1 is an important target to acquire immunity [425], shielding the epitopes might increase the risk of severe disease. We confirmed the previous findings that BgA rosettes hinder the access of antibodies to PfEMP1 [177] and expanded it to all non-O Bgs. Using a small sample set, Goel et al. observed that rosettes formed in A2 were smaller [204]. Moreover, recombinant PfEMP1 domains more readily bind group A1 RBCs [286]. Here, we showed that the durability of rosettes to disruption by antibodies or heparin correlates directly to A-antigen levels on RBCs. To sum up, these findings emphasize that Bg A should be viewed as heterogenous in epidemiolocal studies and possibly BgAB.

PAPER II

“Generation of tools for the study of RIFIN family proteins”.

The RIFIN family has gathered attention for their involvement in immune modulation [211] and BgA rosetting [204]. Rosettes formed in BgA are stronger, more resilient to disruption [414] and shield the pRBC from antibodies [177]. **Paper I** showed that the quantity of BgA on RBCs positively correlates with rosette sturdiness. This is of clinical relevance as individuals with BgA are more susceptible to development of severe malaria [281,285,423,426]. The 150–200 members of the RIFIN family can be divided into A- and B-RIFINSs, which differ substantially with respect to the parasite stage that they are expressed and cellular location [199,207,225,427]. As many studies rely on antibody-based methods, this study aimed to create and validate tools for detection and visualization of RIFINs.

Purified IgG from ten rabbits and one goat, immunized commercially (Agrisera, Sweden) with RIFIN peptides/protein, were characterized in series of assays with a collection of eight laboratory parasite strains. Antigens were designed with the aim of recognizing all RIFINs (C-terminal peptides) or A-RIFINs specifically (indel peptide). First, the ability to recognize a protein of correct size by the antibodies were tested with Western blot using SDS lysates of parasites. Only two rabbit antibodies ($R\alpha RIF_C$ and $R\alpha RIF_I$) and the goat antibody (GαRIF) resulted in bands of the expected size (~35 kDa); in some parasite lines, cross-reactivity was observed with higher molecular weight proteins. Because cellular location can often be indicative of protein function, we probed the antibodies' ability to detect RIFINs by an indirect IFA. The RαRIFC was the only antibody able to stain several parasite strains in a patchy manner distinct from anti-$PfEMP1_{RDSM}$ antibody, which stains the MCs [428]. To determine the exact epitopes of the antibodies produced, an ultra-dense peptide array was used. The array was designed to cover selected members of multigene surface antigen families (PfMC-2TM, PHIST, RIFIN, STEVOR, SURFIN, and PfEMP1) and consisted of 12 amino acid fragments with one residue shift between neighbouring peptides. Four rabbit antibodies and the goat antibody were tested. Two of the rabbit antibodies ($R5\alpha RIF_C$ and $R6\alpha RIF_C$) emerged as unspecific. $R\alpha RIF_C$, $R\alpha RIF_I$, and GαRIF had strong affinity toward the RIFIN that the antigen originated from. Other members of the RIFIN family were also recognized by these antibodies: 141/278 for $R\alpha RIF_C$, 102/278 for $R\alpha RIF_I$, and 66/278 for GαRIF. All antibodies also showed some degree of cross-reactivity against other multigene family members. Finally, the *rif* gene expression in four parasite lines at four timepoints (10, 20, 30, and 40 hpi) was evaluated with RNAseq to verify the RIFIN detection by other methods. RNAseq was not performed for FCR3S1.2 (rosetting) as it had been done before [204], for PAvarO as the genome was not well curated and for 3D7CD36ICAM1 or R29 as the Western blot analysis indicated very low levels of RIFIN expression. Parasites that were negative for RIFINs using RαRIFC antibodies showed low levels of *rif* gene expression. In addition, parasites (FCR3CSA and IT4CD36ICAM) that were positive for $R\alpha RIF_C$ antibodies showed much higher expression of *rif* genes. In fact, the highest expressed RIFINs in those parasite lines match the epitope recognition of $R\alpha RIF_C$ antibodies from peptide array. The peak expression of *rif* genes was found to be at 20 hpi. The results are summarized in Table 1.

In conclusion, six of eight parasite strains probably express RIFINs on their surface. The other two parasite lines presented bands lower than expect with goat antibody in Western blot. Furthermore, no signal was observed for those lines in IFA as well. The inability to detect RIFINs could be due to the parasite expressing a different family member. However, the RNAseq analysis of one of them (NF54CSA) registered only very low levels of *rif* transcripts – i.e., the protein levels might be below detection level. Of eleven animals immunized, only very few resulted in functional antibodies. The RαRIFC antibody against the conserved C-terminal part performed the best overall. It was able to recognize a linear epitope with the band of correct size in Western blot analysis and to detect fixed cellular epitopes on various pRBCs. The peptide array suggests that the likelihood of detection of a randomly expressed RIFIN by this antibody is 50%. However, since the epitope of this antibody is intracellular, it is not

suitable for functional assays such as blocking cellular binding. Goat GαRIF also bound to linear epitopes, but several unspecific bands of higher molecular weight were observed. Moreover, in the peptide array the antibody cross-reacted with some of members of PfEMP1, SURFINs, STEVORs, and PHISTs families. GαRIF antibody has previously been useful in flow cytometry assays with specificity to only one parasite clone (FCR3S1.2R), which might limit its usefulness.

Table 1. Summary of antibodies and assay tested in paper II.

Species	Code	Description of Antigen	Recognizes correct size protein from RIFIN positive parasites line (FCR3S1.2R)	Detects RIFINs from other parasite lines	IFA (patchy staining)	Peptide array
Rabbit	**RαRIF$_C$**	A-RIFIN (PF3D7_0100400), conserved C-terminal peptide (20aa)	Yes[1]	FCR3CSA[1], IT4CD36ICAM1, PAvarO (weak)	FCR3S1.2R, FCR3CSA, IT4CD36ICAM1, PAvarO, R29	141/278 RIFINs including PF3D7_0100400; several SURFINs and STEVORs
	R2αRIF$_C$		No	NT	NT	NT
	R3αRIF$_C$	A-RIFIN (PF3D7_0223100) C terminal peptide (23aa)	No	NT	NT	NT
	R4αRIF$_C$		No	NT	NT	NT
	R5αRIF$_C$	B-RIFIN (PF3D7_0900500) C-terminal peptide (20aa)	No	NT	No staining	Cross-reactivity
	R6αRIF$_C$		No	NT	No staining	Cross-reactivity
	R7αRIF$_C$	B-RIFIN (3D7_0223200) C-terminal peptide(23aa)	No	NT	NT	NT
	R8αRIF$_C$		No	NT	NT	NT
	RαRIF$_I$	A-RIFIN (PF3D7_0100400), semi-conserved indel peptide (25aa)	Yes[1]	FCR3CSA[1], IT4CD36ICAM, PAvarO, S1.2NR	No staining	102/278 RIFINs, including PF3D7_0100400; limited cross-reactivity
	R2αRIF$_I$		No	NT	NT	NT
Goat	**GαRIF**	Full-length A-RIFIN (PF3D7_0100400)	Yes	S1.2NR[3], IT4CD36ICAM1[3], NF54CSA[2,3], 3D7CD36ICAM1[2,3]	Live staining only	66/278 RIFINs including PF3D7_0100400; few members of PfEMP1, SURFINs, STEVORs and PHISTs

NT: not tested
[1]Two bands of similar size (30–50 kDa)
[2] Band smaller than others not recognized by other antibodies
[3]Multiple prominent bands at various molecular weights

PAPER III

"Changes in pH affects the distal aspects of PfEMP1 trafficking onto the pRBC surface".

Pathology of malaria has been linked to PfEMP1-mediated cytoadhesion to various cell types. This family of proteins has been extensively studied in relation to transcriptional regulation and protein trafficking [143,429]. However, these studies are usually done under ideal culture conditions and do not consider changes in the host environment. Upon *in vitro* cultivation of highly rosetting clonal parasite lines unexpected deviations were observed. One of the factors that might cause this was pH. This study aimed to elucidate how PfEMP1 function is affected by pH as local acidification could occur as a result of microvascular occlusion.

To begin with, we determined the rosetting phenotypes of two well characterized laboratory clones (FCR3S1.2 and PAvarO) after 48 h growth in pH-altered media. Rosetting rate, determined using microscopy and flow cytometry, dropped significantly when parasites were grown outside the physiological pH (7.35–7.45). Moreover, the loss of surface detectable PfEMP1 responsible for rosetting followed the same trend over the pH gradient. Next, we explored whether the loss of adherence was rosetting specific or would be expanded to other cytoadhesive phenotypes. Using placental binding NF54CSA parasites, we observed a similar phenomenon, where parasites grown in normal media (pH 7.4) were able to bind more efficiently to placental sections than parasites grown in acidic media (pH 6.8). Yet again, the loss in cytoadhesive capacity was associated with the loss of surface detectable PfEMP1 as determined by flow cytometry. As the antibodies used for PfEMP1 detection were gene-specific and cytoadhesive properties could be changed by switch in *var* gene expression, we collected RNA samples and performed RT-qPCR with dominant *var* gene specific primers. We observed no changes in transcript levels, indicating the observed effect to be independent of switching or downregulation of transcription. These results were confirmed by the recovery of the adhesive phenotype by the following cycle after returning parasites to medium with physiological pH. Immunoblot analysis was performed to investigate possible changes in PfEMP1 total quantity in response to change of pH. No noticeable difference was seen in total cellular PfEMP1 levels. Furthermore, any temporary mis-folding rendering the PfEMP1 unrecognizable to antibodies was ruled out as no differences in immunostaining were detected after 4 h exposure to various pH levels. PfEMP1 is co-transported with other proteins, which could be affected by pH, to form host membrane protrusions. However, scanning electron microscopy images showed no noticeable changes in protrusions. Lastly, mild surface trypsinization was performed to verify the lack of PfEMP1 on the surface on pRBCs. Unique peptides were only detected by mass spectrometric analysis from samples of parasites grown in normal media, and no peptides from samples of parasites grown in acidic media were found.

In this study, we worked from RNA to proteins' surface presentation to identify the cause of decrease in cytoadhesive properties. Findings point toward problems in PfEMP1 trafficking to the pRBC surface in acidified environment. Unlike haemoglobinopathies, which affect the protein transport across PV membrane and lead to altered knob morphology and density [430], here we observed normal protrusions on pRBC indicative of correct formation of subsurface

protein complexes. Hence, the very distal steps of PfEMP1 insertion might be affected. It is also possible that pH-sensitive post-translational modifications play a role in PfEMP1 membrane insertion either directly on PfEMP1 or indirectly on partner proteins needed for correct display. Interestingly, reduced surface display of PfEMP1 was also noted in response to short exposure to febrile temperatures (41 °C) [431]. Although the *in vivo* relevance of the findings in paper III is questionable, it is possible that the loss of cytoadherence at locally acidic microvasculature would allow the pRBCs to move to a more suitable environment. Overall, given the strong association between disease pathology and surface antigens, further studies on the surface display under various host environments are needed.

PAPER IV

"Sevuparin meddles with several aspects of parasite biology".

Several features of *P. falciparum*'s biology make it more virulent than other malaria parasites. These features include promiscuous host cell invasion, efficient parasite multiplication, and excessive cytoadhesion. Although heparin is often used in experimental setups to disrupt rosettes or block parasite invasion, it cannot be used in patients to treat malaria due to its severe side-effects [432,433]. Sevuparin was developed from heparin by removing its anticoagulant activity and has been shown, like heparin, to block parasite invasion and to impede cytoadhesion. Furthermore, it is well tolerated by the patients [21]. This study elaborates on the mechanisms behind the blocking of merozoite invasion and explores other parasite hindering properties of sevuparin and heparin.

Sevuparin can block invasion of all parasite strains/isolates tested, including artemisinin resistant strains [21]. Here, we established additional IC50 values from parasite isolates from South East Asia and South America. The mean IC50 value for all the parasites tested here and during the previous study is 9.5 μg/mL (range 1.8–55.5). To pinpoint the timing of heparinoid (heparin and sevuparin) blocking of invasion, live cell imaging was used. In the presence of heparinoids, merozoites contacted RBCs and remained in contact for an extended period. They were able to weakly deform the RBCs but failed to initiate strong deformations leading to invasion. To identify the possible targets of heparinoids, protein pull-downs from merozoite lysates were done with heparin/sevuparin linked to sepharose beads. Five potential merozoite specific proteins emerged from this analysis: RhopH2, RhopH3, Rap1, Rap2, and Rap3. The targets were further narrowed down using an antibody competition assay. Preincubation with heparinoids prevented the binding of antibodies to the RhopH1, RhopH3, and RhopH complex but not the other merozoite proteins tested. Furthermore, we tested whether peptides designed from RhopH3 could quench the inhibitory effect of sevuparin: one of the peptides containing the predicted heparin-binding site was able to do so. Lastly, we co-incubated recombinant RhopH3 with heparinoids and observed a size shift on the native PAGE gel indicative of heparin binding to RhopH3.

In addition to being essential for invasion, RhopH complex is important for nutrient acquisition as part of PSAC. We observed the parasite development in glucose-limited media as PSAC

inhibition depends on glucose availability [113]. At physiologically more relevant glucose levels (1.2 g/L), we detected a significant delay in schizont development in the presence of heparinoids. This was accompanied with abnormal morphology of trophozoites and increased numbers of extracellular parasites. The schizonts that developed in the presence of heparinoids generated fewer merozoites.

The expression of adhesive virulence proteins could depend on parasite fitness and availability of nutrients. We investigated the effect of heparinoids of PfEMP1 display when added before its appearance on the surface. There was approximately 40% reduction in PfEMP1 on the surface of pRBCs when exposed to heparinoids for extended periods. This is an additional mechanism by which heparinoids can reduce parasite virulence besides interfering with the adhesive properties of already exposed antigens.

As PSAC inhibition by heparinoids was suspected, we performed metabolic profiling of parasites after treatment and compared it to the known PSAC inhibitor furosemide. The principal component analysis of metabolite abundance revealed clear differences between furosemide and heparinoid treatments. This was further confirmed by sorbitol lysis assay where blocking of PSAC prevented osmotic pRBC lysis. As expected, furosemide prevented cell lysis in a dose dependent manner, whereas heparinoids increased the numbers of extracellular parasites.

To identify possible channels and proteins targeted by heparinoids at later developmental stages, protein pull-downs from pRBC ghost lysates were done with heparin/sevuparin-sepharose beads. Several proteins were detected with nanoLC-MS/MS, but none of the members constituted PSAC. Putative interactive proteome was enriched for proteins associated with cation homeostasis, which could explain the haemolysis of pRBCs after heparinoid treatment. Cellular levels of sodium were evaluated using sodium indicative dyes and flow cytometry and by inductively coupled plasma atomic emission spectroscopy. Both methods indicated accumulation of sodium in pRBC specifically after treatment with heparinoids, but no changes were observed for uRBCs. If the elevated in sodium levels result in higher cellular osmolarity, cell swelling and lysis would follow. This could explain the observed increase in extracellular parasites after treatment with heparinoids.

Sevuparin has been put forward as potential fast-acting adjunctive therapy, which could provide time for antimalarials to kill the parasites. This study provided an additional mode of action for sevuparin. To begin with, our investigation of merozoite invasion in the presence of heparinoids points toward heparin inhibition occurring later than previously suggested [54]. Furthermore, it puts forward an idea that the RhopH-complex is the target for heparinoids. The time of invasion inhibition as the result of heparinoids resembles the picture seen when phosphorylation of RhopH3 is prevented [81]. In general, heparinoids inhibit the parasite's ability to multiply by blocking invasion, delaying development, and reducing the number of offspring. Heparinoids also reduce parasite virulence by reducing the surface exposed PfEMP1 levels and blocking the adherence of already exposed proteins. The disturbances in parasite metabolism and growth were suggested to be PSAC independent. Heparinoids might affect

PSAC differently from furosemide giving a distinct metabolic profile or targeting additional channels/transporters on pRBC, which is supported by the increase in sodium levels despite PSAC's low permeability to Na^+ [121].

5 CONCLUDING REMARKS AND POINTS OF PERSPECTIVE

The papers presented here cover various aspects of parasite fitness and virulence. In general, these research findings shed light on parasite biology and offer tools and directions for future studies.

Paper I: We presented an optimized flow-cytometry method that could be a useful tool in many high-throughput studies – e.g., when screening simultaneously for rosette-disruptive agents for adjunctive therapies or when characterizing field isolates. We also showed that all rosettes formed in non-O Bgs reduced the antibody accessibility to PfEMP1, which could contribute to the development of severe malaria. Additionally, we demonstrated a positive correlation between rosette sturdiness and level of A-antigen on RBC surface. This is of utmost importance to future epidemiological and mechanistic studies, which should consider differences between A1 and A weak Bg. Viewing heterogeneous BgA could skew the results and misrepresent the risk odds. From the mechanistic point of view, it would be of interest to test the rosettes of different BgA subtypes under flow conditions in microfluidic channels as BgA rosettes have been shown to be more stable than BgO [434].

Paper II: We demonstrated that different anti-RIFIN antibodies perform differently in various applications. This study highlights the need for good antibody validation before experimental procedures to avoid misleading results. Additionally, use of several antibodies or alternative protein tagging strategies could be employed. Using the antibodies generated, we identified two additional RIFIN expressing parasite lines and confirmed the findings using RNAseq. Future studies can use these RIFIN expressing parasite lines for the investigation of RIFIN functions and potential cytoadhesive properties.

Paper III: Surface display of PfEMP1 on pRBC surface is sensitive to environmental factors such as pH. Therefore, mechanistic studies investigating the PfEMP1 membrane insertion under various conditions are needed. Moreover, as this study focused on PfEMP1, the display of other important surface antigens, such as RIFINs and STEVORs, should also be investigated under changed pH conditions. Understanding how parasites behave in various host environments could help researchers design therapeutics targeting these processes.

Paper IV: We identified possible sevuparin/heparin targets during parasite invasion and discovered additional anti-parasitic properties of sevuparin/heparin. In addition to invasion blocking, the drugs lead to delayed development and reduction in offspring. As parasitaemia is important factor in disease progression, even a small reduction could buy some time for the patients. These drugs were also able to interfere with another important virulence factor: cytoadhesion before and after surface exposure of PfEMP1. The observed effects on fitness and virulence were suggested to be PSAC independent. Further studies are needed that investigate how exactly sevuparin/heparin influences cellular sodium levels. Answering the heparin/sevuparin–sodium conundrum might shade some light on how *Plasmodium* takes advantage of human channels. Overall, sevuparin exhibits good properties for becoming

adjunctive treatment. A recent study proposed that parasite rosetting helped the parasites withstand short artesunate exposures [435]. Hence, the combination treatment with sevuparin would prevent spread of resistant parasites. On the other hand, some studies have seen that disruption of rosettes leads to increased cytoadherence to endothelium, which might worsen the clinical situation [443,444]. Therefore, the benefits and dangers of future treatments should be weighed carefully.

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