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INTERACTION OF *PLASMODIUM FALCIPARUM* ANTIGENS WITH BLOOD GROUP DETERMINANTS: PREFERENCES AND LINK TO DISEASE SEVERITY

BY

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DECLARATION

I hereby declare that this thesis, except for the references to other persons' investigations which I have duly acknowledged, is the result of my own original research and that it has neither in whole nor in part been submitted for another degree elsewhere. This work was done under the supervision of the persons mentioned below.

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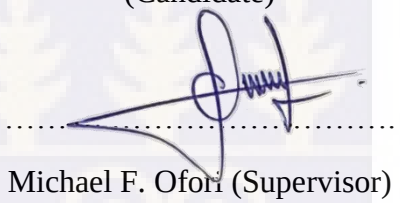

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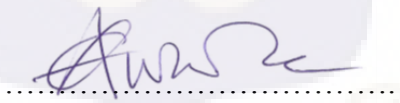

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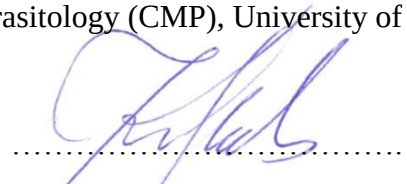

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ABSTRACT

The blood type of malaria patients may determine the outcome of disease. Blood group O associates with protection, while non-O blood groups (A, B or AB) are associated with severe malaria. Rosetting of red blood cells (RBC), which is mediated by blood group A and other red blood cell surface molecules, is also associated with severe disease in the two most virulent species of *Plasmodium* that infect humans. The interaction of parasite ligands with blood group carbohydrates on RBC and endothelial receptors leads to infected RBC sequestration and persistence of the parasite in the vasculature. The objectives of this work were to study and measure the interactions between parasite-expressed factors on the red cell surface and blood group molecules using standard and novel methods, in both laboratory strains and field isolates of *Plasmodium falciparum*. Expression levels of *var* and *rif* transcripts in laboratory isolates selected for binding to blood group antigens were determined, while antibody reactivity to surface antigens were investigated in these isolates. Venous blood samples were collected from 113 children aged 12 years and below diagnosed with malaria and resident in Hohoe, a high malaria transmission zone in the Volta Region of Ghana. The blood type was determined for all patients, while plasma and RBCs were processed and stored for subsequent work.

Long term blood group A and B binding variants of laboratory strains of *P. falciparum* 3D7, FMG and FUP parasites were successfully produced by regular panning on immobilized blood group oligosaccharides, though the production of binding variants in other strains (FCR3 and HB3) were not as successful. Selected parasites were found to bind to both A and B blood group carbohydrates irrespective of the blood group antigen used in selection. The binding isolates also showed a marked adhesion to aorta and dermal endothelial cell (EC) lines. A novel microtiter-plate based quantitative assay to

specifically measure binding of parasite infected RBC to plate-bound blood group antigens was developed and used to determine the interaction of both laboratory and field strains to the blood group A and B antigens. All blood group antigen-selected isolates showed a strong propensity to form rosettes in the presence of RBC from donors of blood type A, AB or B, in contrast to unselected isogenic isolates which did not form rosettes under the same conditions.

The transcription levels of 58 *var* genes of ring stage parasites of 3D7 and FMG selected on blood group antigens and detected by quantitative real-time polymerase chain reaction (QPCR) showed no consistent pattern of expression between selected and unselected parasites, though the adhesion linked genes Pf13_0003 and IT4var32b of 3D7 and FMG, respectively, had elevated levels in some selected isolates.

In conclusion, a novel plate-based assay to directly measure infected erythrocyte adhesion to blood group antigens has been developed. Selection for long term blood group specific parasite binding isolates was also achieved, but with no clear changes in variant surface antigen transcript levels. Notwithstanding, an increased rosette formation and adhesion to non-O blood antigens of selected parasites which suggest a link to severe disease was observed. It is hoped that future investigations using field isolates with clearer categorization of severe cases and a larger sample size would further validate these findings. Whole genome transcriptional studies and sequencing studies in these parasites may also specifically identify the genes and molecules involved in adhesion. Improved knowledge in all these areas will accelerate the design and development of targeted syndrome specific vaccines which will improve the management of this disease.

DEDICATION

To

Naa Aku, Nii Ankrah and *Efua*

for your love and support through this journey

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ARDS	Acute respiratory distress syndrome
ATS	Acidic terminal segment
BD	Becton Dickinson
BSA	Bovine serum albumin
CD	Cluster of differentiation
cDNA	Complementary deoxyribonucleic acid
CIDR	Cysteine-rich interdomain region
CM	Cerebral malaria
CSA	Chondroitin sulphate A
DBL	Duffy-binding like
DC	Domain cassette
DEPC	Diethylpyrocarbonate
EC	Endothelial cell
EDTA	Ethylenediaminetetracetic acid
ELISA	Enzyme-linked immunosorbent assay
EPCR	Endothelial protein C receptor
FACS	Fluorescence –activated cell sorting
FCS	Fetal calf serum
FITC	Fluorescein isothiocyanate
H_2SO_4	Sulphuric acid
Hb	Haemoglobin
HRP	Horseradish peroxidase
ICAM-1	Inter cellular adhesion molecule 1
IE	Infected erythrocyte

Ig	Immunoglobulin
IgG	Immunoglobulin G
IgM	Immunoglobulin M
MACS	Magnetic-activated cell sorting
MFI	Mean fluorescence intensity
NGP	Neoglycoprotein
OD	Optical density
PBS	Phosphate buffered saline
PBS-2	Phosphate buffered saline with 2% FCS
PCR	Polymerase chain reaction
PECAM-1	Platelet endothelial cell adhesion molecule 1
PfEMP1	*Plasmodium falciparum* erythrocyte membrane protein 1
QPCR	Quantitative real-time polymerase chain reaction
RBC	Red blood cell
rif/RIFIN	Repetitive interspersed family
RNA	Ribonucleic acid
SCD	Sickle cell disease
SMA	Severe malarial anaemia
STEVOR	Subtelomeric variant open reading frame
TMB	Tetramethylbenzidine
VSA	Variant surface antigen
vWF	von Willebrand Factor
WHO	World Health Organization

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background

Malaria is a major cause of morbidity and mortality, especially in Sub-Saharan Africa. Figures from the World Health Organization's 2017 World Malaria Report estimates that in 2016, there were 216 million cases worldwide, up 5 million from 2015. This increase is in contrast to the continuous decline in deaths and cases observed over the last decade-and-a-half or so (WHO, 2017).

The disease manifests in several disease forms, and severe forms are defined by clinical or laboratory evidence of vital organ dysfunction. These manifestations can occur singly or, more commonly, in combination in the same patient (WHO, 2012). Clinical features range from impaired consciousness, prostration, convulsions and respiratory distress to acute kidney injury, metabolic acidosis, hypoglycaemia and renal impairment among others (WHO, 2012).

Cerebral malaria (CM), the deadliest form of severe malaria, together with severe malarial anaemia (SMA) account for most of the deaths due to the disease (Murphy and Breman, 2001). Unfortunately, as many as 60% of deaths due to CM are either misdiagnosed or get a delayed diagnosis (Maro and McLarty, 1986). Natural immunity to malaria and protection from severe disease is based on the production of protective antibodies to parasite antigens expressed on the surface of IE during the erythrocytic stage of infection (Chan *et al.et al.*, 2014, Chan *et al.*, 2012, Marsh *et al.*, 1989). These variant surface antigens (VSAs) mediate adhesion to the endothelial membranes,

leading to sequestration of the parasites and avoidance of splenic clearance. In the brain, this contributes to the pathogenesis of CM (Yusuf *et al.*, 2017, Storm and Craig, 2014).

Another of the several life threatening complications of falciparum malaria is SMA, the most common syndrome of severe disease, and a major clinical issue in malaria endemic settings (Lamikanra *et al.*, 2007, Weatherall *et al.*, 2002). Defined as haemoglobin concentrations of less than 5g/dL, a haematocrit less than 15% and parasitaemia greater than 10,000/µL, severe anaemia contributes up to 46% of child fatalities in some developing nations (English *et al.*, 2004). Infants and pregnant women are most at risk for severe anaemia due to malaria, with the risk in infants peaking at 1-2 years of age, depending on the transmission intensity in the area. Prevalence rates of up to 91% in children and 60% to 80% in pregnant women have been reported (Schellenberg *et al.*, 2003, Menendez *et al.*, 2000). SMA is marked by the significant loss of predominantly uninfected RBCs through relatively little understood processes, but known to be influenced by the formation of rosettes, as well as by increased phagocytic clearance (Uyoga *et al.*, 2012). There is also increased haemolysis of both infected and non-infected RBCs, and an abnormal development of erythroid precursors (Abdalla *et al.*, 1980, Casals-Pascual *et al.*, 2006).

Blood groups are complex, inherited chemical systems on blood cell surfaces (and on other cells and body tissues) that determine blood type based on the presence or absence of specific antigens (Kanchan and Krishan, 2016). Currently, there are 35 blood group systems made up of almost 350 of these antigens (Storry *et al.*, 2014, Lane *et al.*, 2016), which may be proteins, carbohydrates, glycoproteins, or glycolipids.

The A and B antigens are both derived from the H determinant precursor to which N-acetylgalactosamine and D-galactose, respectively, are added by glycosyltransferases,

encoded by A and B alleles (Franchini and Lippi, 2015). Type O individuals encode an inactive enzyme that makes no modification to the H determinant, which is their blood group substance. Individuals with different blood types of the ABO group have been known to differ in their general resistance and susceptibility to some diseases (Franchini and Bonfanti, 2015).

In malaria, the relationship between ABO blood group and disease was first noted in 1967 (Athreya and Coriell, 1967), and subsequently there have been arguments to support the idea that the disease has over the millennia, influenced the distribution of these blood groups across the globe (Cserti and Dzik, 2007, Goel *et al.*, 2015, Achur *et al.*, 2008). This distribution indicates a survival advantage for group O compared to others, as the percentage of this group is higher in all malaria endemic areas (up to 90%), which is consistent with selective pressure from the disease (Cserti and Dzik, 2007).

Several clinical studies of severe malaria over the years reflect this advantage of group O over other blood types, with significantly more severe outcomes in the non-O blood groups (Fischer and Boone, 1998, Lell *et al.*, 1999, Pathirana *et al.*, 2005, Tadesse and Tadesse, 2013, Rout *et al.*, 2012, Panda *et al.*, 2011, Tekeste and Petros, 2010). Notably, a meta-analysis of ABO phenotypes and malaria risk in non-pregnant subjects however showed no conclusive evidence associating the blood group phenotypes with the risk of uncomplicated malaria (Loscertales *et al.*, 2007).

The *var* genes of *P. falciparum* encode a group of VSAs referred to as *P. falciparum* erythrocyte membrane protein-1 (PfEMP1) antigens. These are expressed on the RBC surface and were identified as mediators of rosetting (Chen *et al.*, 1998a, Rowe *et al.*, 1997). It is also known that blood group phenotypes modulate *P. falciparum* rosetting,

and subsequently severe malaria (Vigan-Womas *et al.*, 2012). However, the removal or absence of PfEMP1 from IE affected (reduced) rosetting only in blood group O, but not in group A, eliminating PfEMP1 as the only molecule responsible for RBC binding and rosette formation (Goel *et al.*, 2015).

Recent research work has identified the repetitive interspersed family of proteins (RIFINs) as the main molecules involved in non-group O (or PfEMP1 independent) rosetting (Goel *et al.*, 2015). RIFINs are encoded by some 150-200 repetitive interspersed family (*rif*) genes of *P. falciparum*, making them the largest known family of variable IE surface-expressed proteins. They are naturally immunogenic (Abdel-Latif *et al.*, 2002), and a subgroup (A- RIFINs) are exported to the IE surface (Petter *et al.*, 2007).

Only one study so far (Goel *et al.*, 2015) indicates that RIFINs have a preference for binding blood group A antigens on RBC and endothelial cells (ECs), and have been implicated in the pathogenesis of severe *P. falciparum* malaria via rosetting and adhesion to the vascular endothelium.

Initially described as ‘rosettins’ several years ago (Helmby *et al.*, 1993), the functions of RIFINs were still unknown, though they are resistant to enzymatic degradation and known to be up regulated in rosetting parasites (Fernandez *et al.*, 1999, Kyes *et al.*, 1999). Rosette formation has been linked to severe disease, including SMA (Doumbo *et al.*, 2009, Rowe *et al.*, 1995). Being the most commonly observed syndrome of disease severity (English *et al.*, 2004), the identification of RIFINs as mediators in rosetting is a positive step towards better understanding of SMA.

In early studies, a strong association between rosette formation and the ABO blood group was established; rosetting was found to be highest in group A or AB, intermediate

in B and least in group O RBCs (Carlson and Wahlgren, 1992, Udomsangpetch *et al.*, 1993, Rowe *et al.*, 2007). Subsequently, all clinical forms of severe disease were found to have significantly higher rosetting rates (Doumbo *et al.*, 2009), indicating the possibility of targeting the disruption of rosettes as interventions for severe disease.

In *P. vivax* infections, rosetting is frequent and may be linked to the development of anaemia, as the amount of rosetting was higher in anemic individuals (Marin-Menendez *et al.*, 2013).

This study sought to determine, in patients in a malaria endemic setting, differences in the interaction of parasite adhesion factors on IEs in the ABO blood groups, and their link to severe disease. The molecular basis of this was investigated by analysis of real time PCR data from parasites selected for preferences to these blood group antigens. The study in addition, aimed to develop an assay to reliably detect and measure binding between parasites in IE and immobilized blood group antigens *in vitro*.

1.2 Justification and study objectives

1.2.1 Justification

The interactions between parasite IE surface molecules and the ABO blood group antigens have far-reaching implications for therapy and vaccine development in malaria. Though advances continue to be made in drug interventions and vector control that have led to significant reduction in the incidence and mortality rates of malaria over the past two decades, additional tools for accessing these interactions, and ultimately a blood group specific RBC rosette blocking therapy or vaccine (targeting/reducing severe disease) would significantly buttress current efforts to combat malaria where it is most needed. There have been warnings against complacency (Gulland, 2015) following a UNICEF/WHO publication of the reduction (by 50%) in malaria deaths since 2000, and reinforced by the fact that a subsequent report indicated an end to the downward trend of global malaria cases (WHO, 2017). Additionally, there is still a limit to our knowledge of the molecular basis and interactions between parasite phenotypes, their proteins that interact with blood group antigens and how all this affects the clinical outcome of an infection. There is therefore the need, based on previously reported studies, to attempt to further understand some of these interactions and outcomes. The interaction of the infected erythrocyte with host receptors and tissues has over the years focused on the variant surface antigens of the parasite, particularly PfEMP1. More recently, and with the publication of the full parasite genome sequence of *P. falciparum*, the role and significance of some other groups of these antigens have been elucidated, offering a better understanding of host-parasite dynamics and the relationship to disease states and approaches to therapy.

1.2.2 Aims of the study

This study was aimed at going a step further in studying the interaction of parasite infected RBCs with the ABO blood group antigens in particular, by developing a system that is able to specifically measure these interactions, both in the laboratory setting and for use in the field. In addressing these challenges, the study also sought to utilize and adapt existing laboratory isolates for use in this system and to further determine if these changes in phenotype are reflected in transcription of VSA genes previously implicated in blood group specific rosetting.

This would hopefully address hitherto unanswered questions relating to human blood group antigen interactions with this complicated parasite, while providing an alternative way to study particular aspects of the disease. It will also provide, in the long term, further insights to improved therapy and vaccine development approaches that take into account blood group specific differences in the target population. The achievement of all these was based on meeting the objectives of the study listed below.

1.2.3 Specific objectives

- To develop a reliable, quantitative *in vitro* plate-based assay that directly measures the ability of *P. falciparum*-infected erythrocytes to bind human blood group A and B determinants

- To produce and phenotypically characterize *in vitro* adapted and genetically characterized strains of *P. falciparum* with high binding affinity for blood group A and blood group B antigens

  Hypothesis: based on interaction with different host cell surface molecules including blood group antigens, it should be possible to select laboratory strains of *P. falciparum* for binding to blood group antigens in vitro.

- To investigate the expression of 2 major VSA gene families (*var* and *rif*) implicated in the virulence-linked rosetting phenotype through binding to blood group A and B antigens

  Hypothesis: preferential binding by IE to non-O blood type, which is linked to severity of disease, will mirror differences in the VSA gene families that mediate adhesion.

- To investigate the direct binding of field isolates to ABO antigens and test antibody reactivity of patient sera to VSA in parasites selected on blood group antigens

  Hypothesis: There will be field isolates that bind particular blood group antigens, while sera from infected/exposed individuals will differentially recognize parasites selected for binding to different blood group antigens.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 The burden of malaria

The WHO's 2017 World Malaria Report indicates that there were 216 million cases of the disease in 2016, up from the 211 million cases reported in 2015 (WHO, 2017), and ending the decade and a half long trend of declining cases since 2000. The 2000-2015 decline of 60% represented averting (preventing) the deaths of approximately 6.2 million lives that would have been lost to malaria during the period (WHO/UNICEF, 2015a), in addition to an estimated 663 million averted clinical cases (Bhatt *et al.*, 2015). The success chalked in this era was due to the massive expansion in the delivery of core malaria interventions: the distribution of a billion insecticide treated nets (ITNs) in Africa, the introduction of rapid diagnostic tests (RDTs) and the use of artemisinin-based combination therapies (ACT) in treatment (WHO/UNICEF, 2015a). The depiction in Figure 2.1 shows how ITNs represented the most widespread intervention, as well as the largest contributor (68%) of averted cases (Bhatt *et al.*, 2015).

Currently, there are 91 malaria endemic countries, with the top 15 carrying 80% of the global burden, and with a vast majority of cases (90%) in the WHO Africa Region, which also carries 91% of malaria deaths (WHO, 2017). In the African sub region, malaria due to *P. falciparum* accounted for 99% of reported cases in 2016, and was responsible for 91% of the 445,000 malaria deaths recorded globally (WHO, 2017).

In the 2000 to 2015 period, *falciparum* malaria prevalence in endemic Africa was halved, while clinical disease incidence was reduced by 40% (Bhatt *et al.*, 2015). Total

estimated deaths due to malaria were similar for the most recent years, with 446,000 and 445,000 for 2015 and 2016 respectively (WHO, 2017).

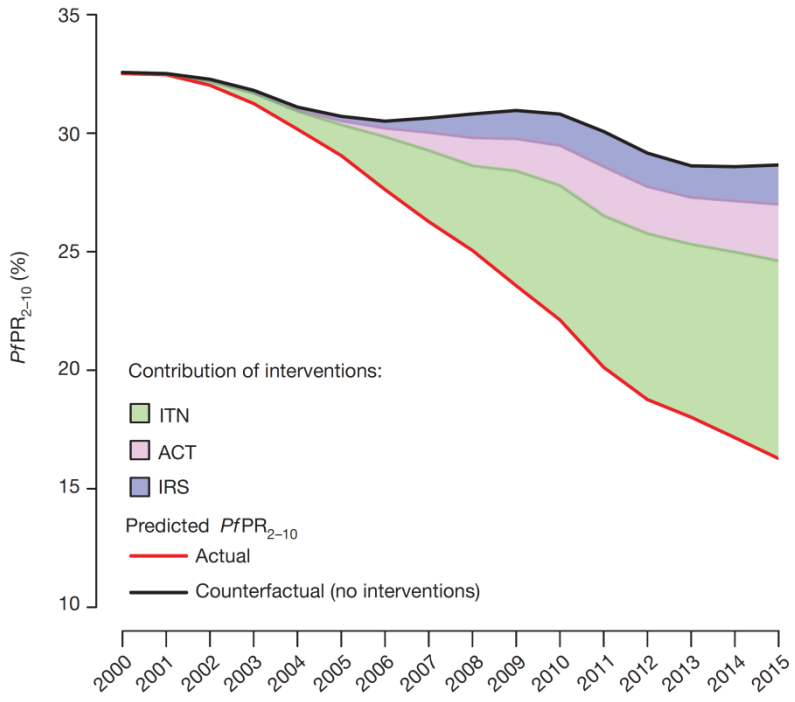


Figure 2.1 **Actual and predicted parasite rates based on interventions**. Geostatistical model-derived predictions (time series of population-weighted mean) of *P. falciparum* parasite rate (PfPR) in endemic Africa. The actual (red line) and predicted (black line) scenario with no ITNs, ACTs or IRS intervention. Coloured areas show relative contribution of each intervention in reducing parasite rate. From Bhatt *et al.* (2015)

Since 2000, over a 15-year period, the children under 5-years global malaria death rate has also fallen by 65 per cent (WHO/UNICEF, 2015a). Though these deaths were almost halved in the 2010 to 2016 period, malaria continues to be a major killer for this age group in high transmission locations, with a child succumbing to malaria every two minutes (WHO, 2018a).

With declining investments in malaria control since 2014, raising the US$ 6.5 billion that will be needed per year by 2020 in order to meet WHO global malaria strategy targets by 2030 will present its own challenges. This figure is far more than what is currently available, and more than twice the US$ 2.7 billion spent in 2016 (WHO, 2017).

These challenges remain, but on the positive side more countries are headed towards malaria elimination. In 2010, 37 countries reported less than 10,000 cases of malaria, the number increasing to 44 countries in 2016. Additionally, the WHO has in 2016 identified 21 countries that could potentially eliminate malaria by the year 2020 (WHO, 2017).

2.2 Malaria: the disease

Malaria in humans is a disease caused by five species of the protozoan parasite of the genus *Plasmodium* and transmitted by the female *Anopheles* mosquito. *Plasmodium falciparum* and *P. vivax* are responsible for the majority of cases and severe forms of the disease, while *P. ovale* and *P. malariae* are less virulent and infect fewer people (White *et al.*, 2014, Antinori *et al.*, 2012). More recently a fifth species of simian origin, *P. knowlesi*, has become a significant cause of severe human malaria in South East Asia (White, 2008, Cox-Singh *et al.*, 2008).

Most infections with malaria take a mild course, mainly with flu-like symptoms including fever, chills, headache, fatigue, nausea, vomiting and muscle pain. However, a significant proportion of patients, mainly children under the age of five years and pregnant women, end up with complications leading to severe disease and fatal outcomes (WHO, 2000a). The observed clinical manifestation of infection with

Plasmodium is the result of responses to the replicating erythrocytic stages of the parasites.

In uncomplicated malaria, the main presentation is fever and mild anaemia, sometimes with an enlarged liver in children, or mild jaundice in adults. Severe falciparum malaria on the other hand may present with additional symptoms such as coma resulting from CM and acidosis irrespective of age, though SMA and hypoglycaemia are more common in children. Figure 2.2 sums up the various facets of pathogenesis of severe disease. In adults, the severe disease symptoms more commonly observed are pulmonary oedema, acute kidney injury and jaundice (White *et al.*, 2014). The majority of deaths from severe disease are attributed to CM and SMA (Murphy and Breman, 2001). Late stages of the parasite (trophozoites or schizonts) are known to sequester in cerebral vessels of CM patients, adhering via surface knobs on IEs (MacPherson *et al.*, 1985).

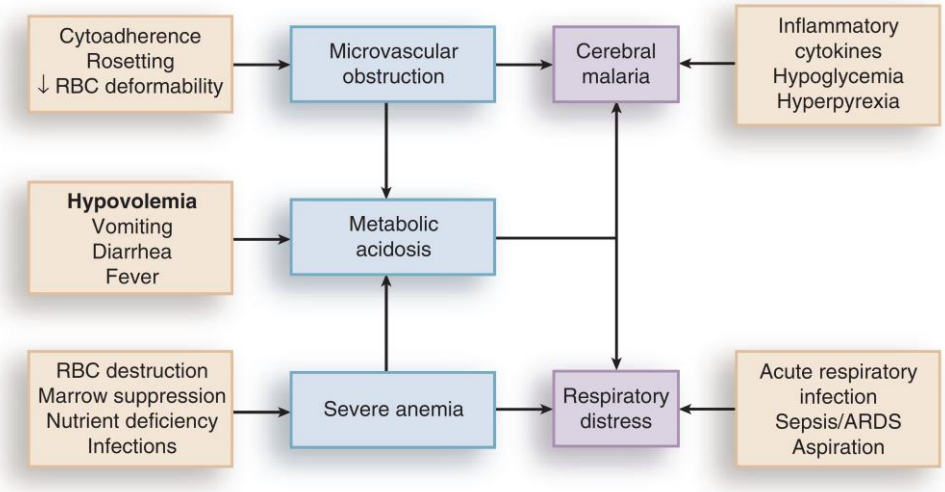


Figure 2.2 **Pathogenesis of severe malaria**. Various factors including host response to infection as well as parasite phenotypes like rosetting and cytoadherence lead to microvascular obstruction, metabolic acidosis and severe anaemia that culminate in CM and other forms of severe disease. ARDS, acute respiratory distress syndrome. From Fairhurst and Wellems (2015)

In Ghana, though some progress has been made over the years in controlling the disease, malaria is still a leading cause of morbidity and mortality, and is also to blame for poverty and low productivity in many areas. In the 2005 to 2015 period, outpatient cases due to malaria in public health facilities reduced by 57%, along with a significant 65% fall in deaths due to malaria. In particular, the number of deaths in children under 5 years of age decreased by 70% in the same period (Aregawi *et al.*, 2017). In line with global objectives, Ghana is targeting 100% of households owning at least one ITN by 2020, and 85% of children under 5 years and pregnant women sleeping under bed nets (https://www.unicef.org/ghana). At the current rate however, the target of 40% reduction in malaria cases and death rates by 2030 will be a challenge to achieve, though interventions such as the intermittent preventive treatment in pregnant women

with sulfadoxine-pyrimethamine, IPTp-SP, have reduced the infection in this group by up to 57% over a 6-year period in parts of the country (Hommerich *et al.*, 2007).

The main cause of malaria in Ghana is *P. falciparum*, and to a much lower extent *P. malariae* and *P. ovale* (Owusu *et al.*, 2017, Kweku *et al.*, 2008). The mosquito vectors known to transmit the disease are *Anopheles gambiae* (*sensu stricto*), which transmits malaria across all the three ecological zones of the country, whereas transmission by *A. melas*, *A. arabiensis* and *A. funestus* is limited to some ecological zones (Duah *et al.*, 2016)

2.3 Malaria: the parasite

Plasmodium parasites are the causative agents of malaria. Transmitted through the bite of female *Anopheles* mosquitoes, this parasite has evolved with humans for millennia, predating the development of modern humans (Cserti and Dzik, 2007). These protozoans belong to the phylum Apicomplexa, which includes several obligate intracellular parasites. There are more than 100 species of *Plasmodium*, infecting a variety of animals including mammals, birds and reptiles (CDC, 2018). Within the highly diverse and successful *Plasmodium* genus, there are 5 species that can infect humans, namely, *Plasmodium falciparum*, *Plasmodium knowlesi*, *Plasmodium malariae*, *Plasmodium ovale* and *Plasmodium vivax* (Keeling and Rayner, 2015).

P. falciparum is the most virulent of these, causing the most severe forms of the disease and a majority of related deaths, quickly turning fatal if not promptly recognized. *P. vivax* and *P. knowlesi* can also cause severe disease (Cox-Singh *et al.*, 2008, Kantele and Jokiranta, 2011), though these are not as common as in *P. falciparum*. *P. knowlesi*

has however emerged as an important cause of human malaria (Singh *et al.*, 2004), and in parts of Southeast Asia is now the most common cause of the disease (William *et al.*, 2013, Yusof *et al.*, 2014). More recently there are calls to acknowledge the importance of *knowlesi* malaria in the WHO's World Malaria Report so as to increase identification and improve monitoring of the parasite (Barber *et al.*, 2017).

2.3.1 The Life Cycle of *Plasmodium* Species

The life cycle of *Plasmodium* species is complicated, consisting of an exogenous sexual phase (sporogony) and an endogenous asexual phase (schizogony) that occur respectively in the female anopheles mosquito and vertebrate hosts. In humans (Figure 2.3), infection begins with the injection of sporozoites, along with the saliva of an infected mosquito during its blood meal, into the dermis of the skin. Sporozoites enter the circulation and are quickly transported to the liver where they migrate through several hepatocytes, finally settling into one, within the parasitophorous vacuole of the parasite (Mota *et al.*, 2002). Here, it differentiates and multiplies several times mitotically, up to 40,000 merozoites per hepatocyte (Cowman *et al.*, 2016), which are released into the blood stream via vesicles (merosomes) that bud from the hepatocyte (Sturm *et al.*, 2006). The length of this pre-erythrocytic stage is species dependent, ranging from between 5-7 days in *P. falciparum* up to 14-16 days in *P. malariae* (Antinori *et al.*, 2012). The release of merozoites from hepatocytes marks the end of the pre-erythrocytic stage and the beginning of the asexual erythrocytic phase (blood stage) of the life cycle.

Free merozoites very quickly invade red blood cells, the process taking only 1-2 minutes to complete (Paul *et al.*, 2015). Successful merozoite invasion of RBC is

dependent on the recognition, attachment and entry into the erythrocyte. Initial contact may occur anywhere on the merozoite surface, followed by re-orientation of the parasite to enable alignment of the apical end to the entry point on the RBC membrane to form a tight junction (Cowman and Crabb, 2006). This tight junction, powered by the actin-myosin motor of the parasite, moves from the apical to the posterior end of the merozoite, pushing the parasite into the RBC cytoplasm. At the same time, this action forms a parasitophorous vacuole around the merozoite (Cowman and Crabb, 2006, Keeley and Soldati, 2004).

Within the RBCs, the early ring stages mature through trophozoites into late schizont forms over a 48-hour period. RBCs with these late stages eventually rupture to release between 16 and 36 merozoites per RBC, producing a new wave of the asexual blood stage cycle (Cowman *et al.*, 2016, Antinori *et al.*, 2012). All clinical symptoms attributed to malaria manifest during this stage of the disease.

During the erythrocytic cycle, some merozoites develop into male and female gametocytes, which are sexual forms of the parasite capable of being picked up by the vector during feeding. Gametocytes sequester and develop in the bone marrow, reappearing in the peripheral circulation when mature (Cowman *et al.*, 2016).

In the mosquito gut, gametocytes develop into gametes and fuse to form zygotes. The zygote develops into an ookinete and then an oocyst in the mosquito gut. The mature oocyst then bursts to release sporozoites that migrate to the salivary glands of the mosquito to complete the cycle during the next blood meal.

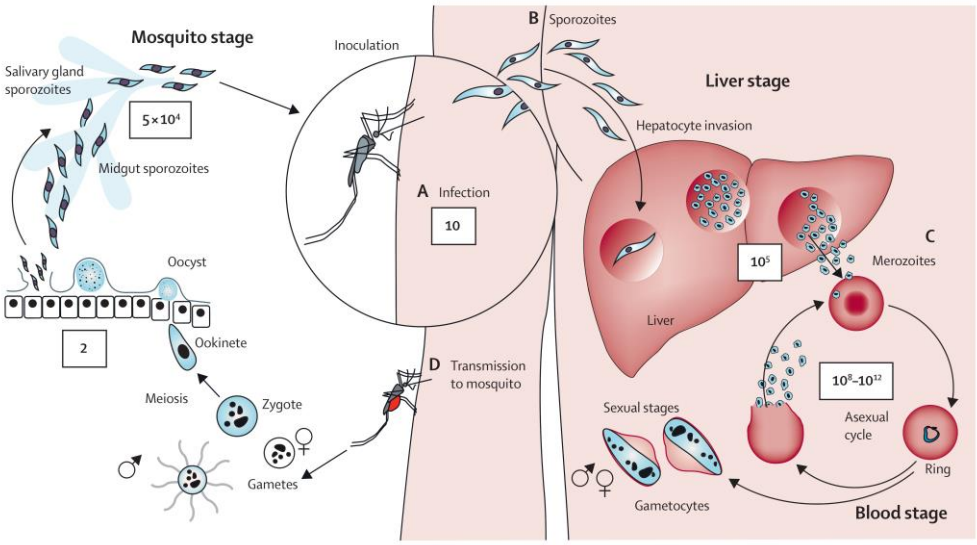


Figure 2.3 **The life cycle of *P. falciparum* in the human and Anopheles mosquito vector**. Sporozoites in saliva are injected into the skin at bite site (A), migrate to the liver (B), and invade hepatocytes. Here, they multiply and burst out of the liver cells and invade RBCs, marking the beginning of the asexual cycle (C) of merozoite growth and maturation to trophozoites and schizonts. Some parasites form gametocytes (sexual forms) that are taken up by the vector during a blood meal (D). In the mosquito gut they mature into gametes and reproduce sexually resulting in an ookinete, eventually resulting in an oocyst full of sporozoites, which burst out and migrate to the salivary glands. White boxes show approximate number of parasites for that stage. From White *et al.* (2014).

2.3.2 Multigene families of *P. falciparum*

During the asexual stages of malaria, *P. falciparum* modifies the infected erythrocyte by inserting highly polymorphic variant surface antigens (VSAs) that undergo clonal antigenic variation (Roberts *et al.*, 1992, Brannan *et al.*, 1994) in the membrane. The parasite has 14 chromosomes with a haploid nuclear genome of about 23 Mb. The genes that code for the variant antigens associated with the IE membrane are mostly located in

the subtelomeric regions of the chromosomes (Gardner *et al.*, 2002), with the 3 main variant gene families; the *var* family of genes which encode the *P. falciparum* erythrocyte membrane protein 1 (PfEMP1), the repetitive interspersed family, *rif*, that code for RIFINs, and the subtelomeric variant open reading frame (*stevor*) genes that code for STEVOR proteins, all located in subtelomeric block 4 of the five unique subtelomeric blocks. This region contains all the *stevor* genes, 36 of the 59 *var* genes and 149 of the 159 *rif* genes (Rasti *et al.*, 2004). Apart from the three mentioned before, there are other lesser studied VSAs such as the SURFIN protein family, which are encoded by a group of 10 surface-associated interspersed genes (*surf* genes) and have structural and sequence similarities with other exported surface antigens (Winter *et al.*, 2005).

These VSAs serve an important role by facilitating parasite cytoadhesion to vascular ECs of the host, thereby avoiding clearance in the spleen and contributing to the pathogenesis of malaria (Berendt *et al.*, 1994). They also serve as an immune evasion strategy due to the high turnover in protein sequence, making it difficult for the host immune system to keep up with the changing antigens presented. The immune response to the blood stage is primarily targeted at VSAs (Staalsoe *et al.*, 2003), and antibody reactivity to these has been consistently linked to disease severity and conditions, and also to protection against clinical malaria (Chan *et al.*, 2014, Chan *et al.*, 2012, Marsh *et al.*, 1989). For example, VSA antibody levels were found to correlate positively with the age of healthy children, but negatively with that of malaria patients (Ofori *et al.*, 2002). Similarly, fewer VSAs were recognized by sera from children with SMA compared with sera from children with CM, while sera from those with severe disease were generally less reactive to several of the RIFIN and STEVOR VSA families (Travassos *et al.*, 2018).

2.3.2.1 The *var* genes

The *var* family of genes, constituting a family of about 60 copies per genome, encode a group of VSAs termed *P. falciparum* erythrocyte membrane protein-1 (PfEMP1). These large molecules (250-350kDa), exported to and expressed on knobs on the surface of IE (Oh *et al.*, 2000), are the best characterized member of the VSA, and known to mediate cytoadhesion to endothelial receptors of the host (Baruch *et al.*, 1995, Gardner *et al.*, 2002, Su *et al.*, 1995, Treutiger *et al.*, 1997, Newbold *et al.*, 1997b). As well as mediating sequestration, clonal antigenic variation within this family of genes enables the parasite evade host antibody responses (Kyes *et al.*, 2001). The parasite also exhibits allelic exclusion, which means that usually only one of these *var* genes is expressed at a time (Chen *et al.*, 1998b, Scherf *et al.*, 1998), enhancing its host immune evasion capabilities. The subtelomeric location means some *var* genes are more vulnerable to recombination, while centrally located *var* genes are relatively conserved, though recombination can affect their stability (Gardner *et al.*, 2002).

Structurally, PfEMP1 molecules are composed of several distinct domain structures. The extracellular component is made up of an N-terminal segment (NTS) which is variable, followed by several Duffy-binding-like (DBL) domains and cysteine-rich inter-domain regions (CIDRs). The more conserved intracellular acidic terminal segment (ATS) is the C-terminal component that may play a role in anchoring the molecule to erythrocyte skeletal proteins (Oh *et al.*, 2000), and is linked to the extracellular component via a transmembrane domain (Rasti *et al.*, 2004), shown in Figure 2.4. There are 4 main groups (A, B, C, and E) of PfEMP1 based on the *var* gene sequence that encodes the proteins (Smith, 2014). There are variants of PfEMP1 classified based on the domain cassette (DC) system consisting of certain DBL and CIDR domain structure combinations. Parasites with specific DCs (DC8, DC13) are

associated with binding to the vascular endothelium of the brain and thus to severe malaria in children (Claessens *et al.*, 2012, Avril *et al.*, 2012, Lavstsen *et al.*, 2012).

As the primary parasite protein mediating cytoadhesion (Pasternak and Dzikowski, 2009), PfEMP1 binds to a variety of host endothelial receptors including cluster of differentiation 36 (CD36), chondroitin sulphate A (CSA), PECAM-1, inter cellular adhesion molecule 1 (ICAM-1) and endothelial protein C receptor (EPCR), the latter two of which are expressed on brain microvascular ECs. Figure 2.4 shows a schematic depiction of the domain structure of PfEMP1 showing the group structure and some of the corresponding host receptors mentioned.

EPCR is a host receptor important in the cytoprotective protein C pathway for regulating blood clotting and for endothelial barrier integrity (Bouwens *et al.*, 2013). The binding of PfEMP1 may interfere with this pathway, resulting in blood-brain barrier dysfunction and pathological damage of the endothelium (Moxon *et al.*, 2013). However, the ultimate outcome of disease could well depend on the specific domain compositions of the PfEMP1 variant in question (Smith, 2014).

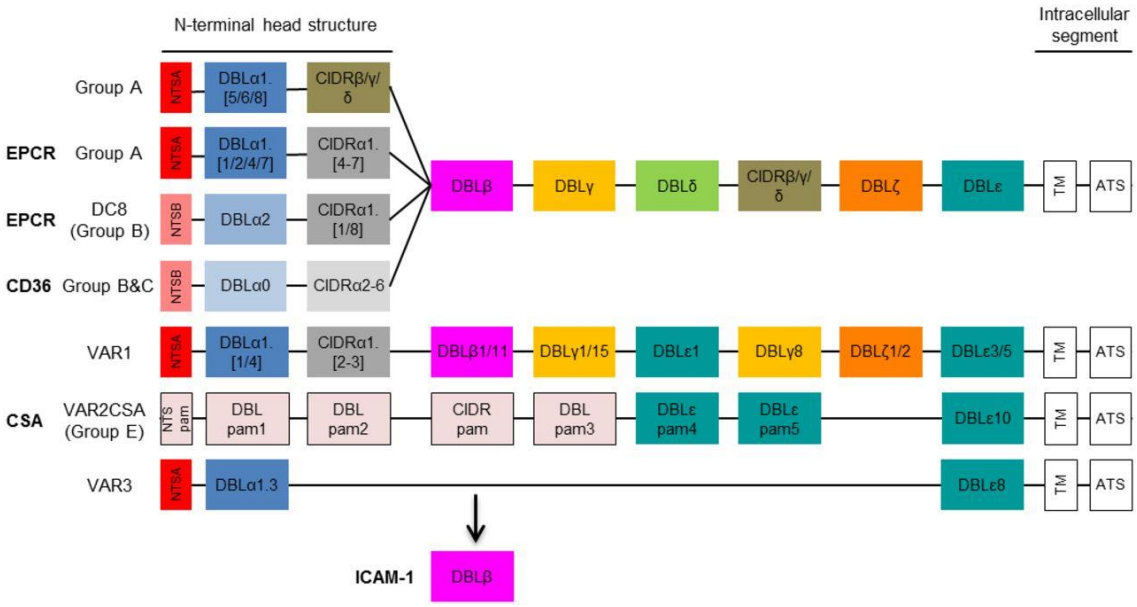


Figure 2.4 **The domain structure of PfEMP1.** Structurally, PfEMP1 consists of a hypervariable, extracellular N-terminal head structure and 2 to 6 subsequent domains of Duffy binding like (DBL) or cysteine-rich interdomain region (CIDR), a transmembrane domain (TM) and an intracellular acidic terminal segment (ATS). Receptors are indicated in bold. Figure reproduced from Storm *et al.* (2019)

2.3.2.2 The *rif* genes

With 160 copies per haploid genome (Zhou *et al.*, 2019), RIFINs are the largest and most diverse multigene family in *P. falciparum*, but have not been as well studied or characterized as the *var* genes, having only recently generated much research interest (Ch'ng *et al.*, 2017). First described by Kyes *et al.* (1999), RIFINs consist of two sub-groups, most (70%) belonging to the group possessing a 25 amino acid insertion-deletion (indel) region (subgroup A, or A-RIFINs), and subgroup B (B-RIFINs) that lack an indel (Joannin *et al.*, 2008). These subgroups also differ in their localisation, with A-RIFINs shown to be exported to the host cell membrane while B-RIFINs remain

within the parasite (Haeggstrom *et al.*, 2007, Bachmann *et al.*, 2012, Petter *et al.*, 2007, Joannin *et al.*, 2008). Importantly, and unlike the *var* genes, several *rif* genes are simultaneously transcribed in the parasite, with multiple proteins expressed at the Maurer's clefts (MCs) and on the IE surface (Kyes *et al.*, 1999, Fernandez *et al.*, 1999, Khattab and Klinkert, 2006). These 27-45kDa membrane proteins seem to be expressed in all stages of the parasite in the human host, though their functions at these stages are not fully known (Petter *et al.*, 2007, Wang *et al.*, 2009, Mwakalinga *et al.*, 2012). Along with other VSA genes like the *var* and *stevor*, *rif* genes cluster mainly in the subtelomeric regions of all 14 chromosomes of the parasite (Gardner *et al.*, 2002).

RIFINs may serve as potential vaccine candidates, mediating blood group A rosetting and believed to influence blood group distribution, all of which has implications in disease control and management (Goel *et al.*, 2015, Tan *et al.*, 2016). A study by Abdel-Latif *et al.* (2003) in a comparison of antibody responses to RIFINs between immune adults and semi-immune children, reported much higher recognition by the immune adults, indicating that naturally acquired antibodies target these RIFINs. In addition, the fact that removal of recombinant RIFIN-adsorbed antibodies reduced reactivity to VSAs in the plasma of immune individuals point to the contribution of naturally acquired anti-RIFIN antibodies to VSA antibody responses (Abdel-Latif *et al.*, 2004).

2.3.2.3 The *stevor* genes

Another major gene family of *P. falciparum* are the *stevors*, with some 30 copies encoding antigenically variant STEVORs of 30-40kDa (Zhou *et al.*, 2019). Initially thought to be contained within the MC (Kaviratne *et al.*, 2002), it was eventually demonstrated that STEVOR moves out of the MC and is expressed on the IE surface

(Niang *et al.*, 2009), thus playing a role in the antigenic variation of late stage parasites. STEVORs, along with RIFINs, are involved in the pathogenesis of malaria and immune evasion, though the knowledge on the protection offered by antibodies to these two VSA families is not extensive (Yam *et al.*, 2017, Wahlgren *et al.*, 2017). In contrast to patient isolates however, STEVOR expression in culture adapted parasites is very low, an indication that this protein is not necessary for survival in culture. STEVORs also mediate rosetting (Niang *et al.*, 2014), which is presumed to protect against invasion-blocking antibodies that would otherwise limit merozoite attachment and erythrocyte invasion.

2.4 The ABO blood group system

The human ABO blood group system in red cells was discovered over a century ago by Karl Landsteiner. These carbohydrate antigens are widely distributed throughout the body. They can be found in saliva, gut, endothelia of blood vessels as well as on the epithelial cells of some tissues, particularly those in direct contact with the environment such as in the trachea, nose and in the urinary, respiratory and reproductive tracts (Ravn and Dabelsteen, 2000, Eastlund, 1998, Szulman, 1960). Though found in the tissues of many animal species, the presence of the ABO blood group antigens on the RBC is unique and limited to humans and some apes (Oriol *et al.*, 1986).

Blood group antigens, which are terminal structures of glycan chains, are synthesized by the action of glycosyltransferases encoded by the *ABO* gene that sequentially add monosaccharide units to a variety of disaccharide precursors. To produce the H antigen, an α1,2 fucosyltransferase catalyzes the addition of a fucose in α1,2 linkage to the precursors. In humans, two α1,2 fucosyltransferases encoded by the *FUT1* and *FUT2*

genes are involved. Mutations in these genes lead to the Bombay (absence of ABH antigens on RBCs) and non-secretor phenotype (absence of ABH antigens in saliva and epithelia) respectively (Marionneau *et al.*, 2001). Following biosynthesis of the H antigen, the subsequent addition of an N-acetylgalactosamine in α1,3 linkage will yield blood group A antigen, while the addition of D-galactose at the same position yields blood group B antigen.

Two of the three main alleles of the blood group system, *A* and *B*, are co-dominant while the *O* allele is recessive, the combinations of these resulting in the four major phenotypes (A, B, AB and O) observed in humans (Yamamoto *et al.*, 1990).

The biological significance, and therefore the driving force for the evolution of the ABO system in humans is not fully elucidated, though pathogens have been suggested as the cause due to several associations (Seymour *et al.*, 2004, Frattali Eder and Spitalnik, 1997). Some studies suggest the modulation of cellular interactions on the RBC surface by ABH antigens to stabilize glycan molecules as an alternate explanation for this evolution (Cohen *et al.*, 2009). Blood groups have been associated with susceptibility to several diseases including tropical infections caused by parasitic helminths like schistosomiasis (Kassim and Ejezie, 1982) and onchocerciasis (Opera, 2007), as well as viral infections like HIV (Abdulazeez *et al.*, 2008). However, in some of these instances, there has been no clear-cut or conclusive proof of such associations (Anstee, 2010). Malaria is no exception, and associations between the disease and blood groups are well known (Fry *et al.*, 2008, Rowe *et al.*, 2007, Miller *et al.*, 2002). In a Ghanaian study, it was observed that patients with complicated cases of malaria were twice as likely to be of non O blood groups, and for blood group O patients with complicated disease, parasitaemia was low (Afoakwah *et al.*, 2016).

There are also studies that link blood groups to genetic blood disorders such as haemoblobinopathies and thalassemias. In sub Saharan Africa, sickle cell disease (SCD), recognized as a problem of major public health significance (WHO, 1994), is linked to decreased susceptibility to malaria (Awotua-Efebo *et al.*, 2004, Aidoo *et al.*, 2002), also for the heterozygous (AS) individuals (Williams *et al.*, 2005, Amodu *et al.*, 2012). In a study in Nigeria, blood group O was the blood type most commonly associated with the SS genotype (Alagwu *et al.*, 2016). Interestingly, in another Nigerian study, SCD patients with non-group O blood types presented with almost double the relative risk and significantly higher vaso-occlusive crisis episodes compared to blood group O SCD patients (Ahmed *et al.*, 2014).

Some studies indicate that the most common blood group observed in β-thalassemia is group O+, and though this group also shows the most complications of the disease, they occur much later in life and are milder in severity compared to other blood types (Sinha *et al.*, 2017, Marbut *et al.*, 2018).

2.5 Immunity to malaria

Immune response to the *Plasmodium* species, though studied for many decades, is still unclear and remains challenging in its complexity. The importance of antibodies in protection has been investigated in several species (Brown, 1969, Miller *et al.*, 1975, Mitchell *et al.*, 1976). In humans, the importance of humoral immunity was demonstrated in a classical study where passive transfer of purified IgG antibodies from

immune individuals to patients with malaria resulted in parasite clearance and recovery (Cohen *et al.*, 1961).

Clinical immunity to malaria develops slowly and only after repeated episodes over time following recurrent infections with *P. falciparum* in holoendemic areas, with adults eventually becoming protected from clinical disease (Bull *et al.*, 1998, Marsh *et al.*, 1989) as antibodies are acquired from successive exposure to the repertoire of surface antigens (Giha *et al.*, 2000, Contamin *et al.*, 1996). This immunity is acquired in a systematic manner, with protection first acquired from the most severe forms of the disease in the children, followed by protection from less severe, uncomplicated malaria as they grow (Hviid, 2005, Gupta *et al.*, 1999). Generally, asyptomatic *P. falciparum* infections associate with decreased incidence of clinical disease, but offer no protection in new infections (Buchwald *et al.*, 2019). Immunity however is not directly linked to age, since adults with no prior exposure to the disease will develop severe disease (Barcus *et al.*, 2003). Studies suggest that protective antibody responses in *falciparum* malaria are directed primarily to the parasite encoded exported PfEMP1 variant antigens on the IE surface (Bull *et al.*, 1998, Giha *et al.*, 2000, Dodoo *et al.*, 2001).

Studies have shown associations between specific IgG antibody classes and subclasses that are associated with protection and those that are not (Bouharoun-Tayoun and Druilhe, 1992, Domingos *et al.*, 2018). Studies have also indicated the protective role IgM may have in malaria, with lower levels in nonimmune individuals and a negative correlation with parasite densities observed (Boudin *et al.*, 1993, Wahlgren *et al.*, 1986). Anti-malarial IgE on the other hand, which increase during *P. falciparum* infection, has been linked both to reduced risk from clinical disease (Bereczky *et al.*, 2004, Duarte *et al.*, 2007) and to severe disease (Perlmann *et al.*, 1994, Calissano *et al.*, 2003).

The VSAs consisting of exported parasite proteins mediate the virulence-linked mechanisms of cytoadhesion/sequestration and rosetting through products of the 3 main protein multigene families represented by PfEMP1 (Smith *et al.*, 2013), RIFIN (Goel *et al.*, 2015) and STEVOR (Niang *et al.*, 2014) proteins, which are attributed to the cause of severe malaria (Ringwald *et al.*, 1993).

2.5.2 Sequestration of *P. falciparum*

Sequestration is described as the adhesion of late-stage *P. falciparum*-infected erythrocytes to ECs (Udeinya *et al.*, 1981), primarily within the venules. This ability is critical to the organism as it enables the parasite to avoid clearance by the spleen. In addition, the hypoxic venular environment with reduced oxygen tension is ideal for the development of the parasite (Scheibel *et al.*, 1979). The brain, heart, lung, liver, kidney, subcutaneous tissues and placenta are all sites where *P. falciparum*-infected erythrocytes have been found to be sequestered (Miller, 1969, Yamada *et al.*, 1989). Sequestration of IE in the brain microvasculature is the histopathological indicator of CM, which can lead to obstruction of these vessels with fatal consequences (Milner *et al.*, 2015). Apart from the mechanical obstruction caused by rosetting and cytoadherent RBCs (Chen *et al.*, 2000a), sequestration also induces pathology due to hyper inflammatory host responses leading to blood-brain barrier dysfunction and cerebral edema (Brown *et al.*, 1999). Other complications including acidosis, respiratory distress and SMA may be indicated (Rowe *et al.*, 2009).

Host receptors for cytoadhesion are numerous and varied, and includes the intercellular adhesion molecule 1 (ICAM-1). Expressed on the endothelium and several immune cells, this receptor is one of the most well described receptors for PfEMP1. ICAM-1 is upregulated following adhesion of IE and in response to pro-inflammatory cytokines

(Berendt *et al.*, 1989, Chakravorty and Craig, 2005). In CM cases of severe malaria, this sequestration occurring via ICAM-1 mediated adhesion results in pathology *in vivo*, with histologically demonstrable co-localization of ICAM-1 and IE in the brain ECs of patients who succumbed to the disease (Turner *et al.*, 1994). In addition, other studies show higher adhesion to ICAM-1 in parasites from severe disease, than in those from uncomplicated disease or controls (Ochola *et al.*, 2011, Newbold *et al.*, 1997a).

The common membrane protein CD36 which is expressed on the vascular endothelium and several other cells such as monocytes, macrophages, dendritic cells and platelets (Silverstein and Febbraio, 2009) is also a known receptor for *P. falciparum* cytoadhesion. CD36 binding is fairly common in most *P. falciparum* strains (Turner *et al.*, 1994, Rowe *et al.*, 2009, Newbold *et al.*, 1997a), and is also linked to the phagocytosis of IE (McGilvray *et al.*, 2000). There is however no clear role for CD36 in the pathogenesis of malaria, as there has been no clear-cut association to disease severity (Rogerson *et al.*, 1999, Newbold *et al.*, 1997a).

2.5.3 Red blood cell rosetting in malaria

Rosetting was first described as a spleen-related property by David and colleagues in 1988, defined as the adhesion of uninfected RBC(s) to an infected one, or 'the agglutination of uninfected erythrocytes around parasitized erythrocytes' (David *et al.*, 1988). This phenomenon involves mature or late stage parasites of certain strains of *P. falciparum* (Handunnetti *et al.*, 1989), and has not only been described in all species known to infect humans (Lowe *et al.*, 1998) such as *P. vivax* (Udomsangpetch *et al.*, 1995, Chotivanich *et al.*, 1998) and *P. ovale* (Angus *et al.*, 1996), but also in some

simian (Udomsangpetch *et al.*, 1991) and murine (Mackinnon *et al.*, 2002) malaria parasites.

The ability of IEs to adhere to uninfected ones and to the endothelium is thought to be crucial to the pathophysiology observed in CM, SMA and other syndromes of severe malaria (Sherman *et al.*, 2003, Miller *et al.*, 2002, Carlson *et al.*, 1990). Rowe and colleagues for example reported significantly lower rosetting rates in milder forms of disease, with increasing rates as severity increased (Rowe *et al.*, 1995). In this same study, rosetting frequency of isolates from donors of blood group O was less than rosetting observed in non-group O (A and AB) isolates, while in another study significantly high levels of rosetting was observed in all clinical forms of severe disease including coma, SMA, neurological impairment and seizures (Doumbo *et al.*, 2009). Other studies have demonstrated that more pronounced rosetting is observed in isolates from severe malaria patients, particularly those from cases with CM (Treutiger *et al.*, 1992, Rowe *et al.*, 1995, Ho *et al.*, 1991). In addition, rosetting has been shown to enhance vaso-occlusion (Kaul *et al.*, 1991), but the relation between rosetting and virulence remains unclear, since rosetting is also observed in other, less virulent, malaria species (Rowe *et al.*, 2009).

The changes occurring on the surface of IEs stem from extensive modifications made by the parasite, which exports proteins to the erythrocyte surface, making sequestration possible by binding to host endothelial receptors. The biological role or functional significance of rosetting *per se* is not yet clear, though there may be pathogenic implications such as occluding capillaries, enhancing reinvasion due to proximity of uninfected erythrocytes, physically blocking immune attack on IE and even a host defense mechanism to discourage cytoadhesion (Wang and Hviid, 2015). Conversely,

rosetting may also reflect a bystander effect to EC adhesion in which RBC happen to share common receptors with the ECs in question.

PfEMP1, RIFIN and STEVOR proteins all mediate the rosetting, previously thought to be mediated by PfEMP1 alone. *P. falciparum* RIFINs were shown to preferentially bind to blood group A (and perhaps B) antigens on the erythrocyte surface, making them the parasite ligand primarily responsible for rosetting and microvascular binding in non-group O blood types. This interaction seems to result in larger rosettes and increased binding of IEs in the microvasculature of individuals with blood group A (Carlson *et al.*, 1994), rosettes that are also more resistant to heparin-induced rosette disruption (Ch'ng *et al.*, 2016). These properties of non-group O (specifically group A) rosettes, in addition to the fact that they shield IE from antibody binding (Moll *et al.*, 2015), ultimately implicate them in disease severity. STEVOR on the other hand was found to mediate rosetting independent of PfEMP1 through Glycophorin C (GPC) on the RBC surface (Niang *et al.*, 2014).

In a 2005 study, it was reported that a novel group of parasite derived polymorphic surface proteins, the surface-associated interspersed gene family (SURFIN) proteins (Winter *et al.*, 2005), may also have a role in rosetting, either directly or as an accessory molecule (Quintana *et al.*, 2018).

IgM was thought to mediate rosetting through Fc receptor binding to IE and therefore affect disease severity (Rowe *et al.*, 2002, Ghumra *et al.*, 2008), but subsequent studies indicate that this IgM binding does not directly mediate the formation of rosettes, though it may, along with α_2- macroglobulin, be important for some forms of the phenomenon (Stevenson *et al.*, 2015a, Stevenson *et al.*, 2015b, Akhouri *et al.*, 2016). Also, complement receptor 1 is considered a malaria resistance gene, as polymorphisms that reduce CR1 on the RBC reduce rosetting and protect from severe disease

(Cockburn *et al.*, 2004). Understanding the phenomenon of adhesion and rosetting, along with the ligands and receptors involved in the development of severe disease, has huge implications in disease management and possible drug and vaccine targets.

2.6 Malaria vaccines

There have been significant declines in malaria morbidity and mortality in the past decade or so, but the global burden of malaria remains unacceptably high. Of concern as well is the fact that for the parasite, there are reports of development of resistance to the first line drug Artemisinin (Noedl *et al.*, 2008), as well as the observation that mosquitoes may be changing feeding behavior to adapt to pressures from large scale ITN coverage (Russell *et al.*, 2011). A highly effective malaria vaccine is very much needed to support the efforts in worldwide malaria control, and would be critical for total elimination of the disease in the most affected areas (Laurens, 2018). This could provide at least some level of immunity to subjects (at best, sterilizing immunity), while buttressing current successes chalked in ITN schemes, vector management and ACTs (WHO, 2018b).

There is currently just one malaria vaccine, RTS,S/AS01 (also known as Mosquirix™), approved in 2015 by the European Medicines Agency for use in children in malaria endemic countries (Gosling and von Seidlein, 2016). Unfortunately efficacy is limited, and protection is also short-lived for this vaccine, especially in infants (White *et al.*, 2015). There are however suggestions that this vaccine may additionally prove to be beneficial in transmission interruption efforts in areas of low-endemicity (Gosling and von Seidlein, 2016).

Placental malaria, which results from *P. falciparum* infection in pregnant women, is characterized by the sequestration of IE in the intervillous spaces of the placenta where they bind to CSA through a PfEMP1 variant VAR2CSA (Duffy *et al.*, 2006, Clausen *et al.*, 2012). This results in inflammation and other negative outcomes including perinatal mortality, low birth weight and premature births (Rogerson *et al.*, 2007, Fried and Duffy, 2017) Immunity to these placental parasites, like other forms of malaria, is achieved over successive pregnancies (Ricke *et al.*, 2000, Doritchamou *et al.*, 2019b), which has made VAR2CSA a potential vaccine candidate for malaria in pregnancy. Clinical trials of two VAR2CSA vaccine candidates designed to block IE binding to CSA; PAMVAC (Mordmuller *et al.*, 2019) and PRIMALVAC (Sirima *et al.*, 2020), are underway, and results of some studies underscore the need for targeted vaccines based on parasite, individual or geographical differences (Doritchamou *et al.*, 2019a)Antibodies have long been known as important for protective immunity against malaria, as shown in classical passive transfer studies (Cohen *et al.*, 1961). Unfortunately, the generation of these protective antibodies by vaccination has been challenging, as definite correlates of immunity have been difficult to ascertain (Fowkes *et al.*, 2010). There have been several different subunit malaria vaccines developed to clinical testing stages, but results from these have not been encouraging (Thera and Plowe, 2011, Chuang *et al.*, 2013, Ogutu *et al.*, 2009, Hodgson *et al.*, 2014, Genton *et al.*, 2002). However, recent trends point to a growing interest in whole parasite vaccines targeting the liver stage to block infection (Laurens, 2018)

With reports of developmental advances in several areas of malaria vaccine research, from infection-blocking pre-erythrocytic vaccines, to infection- and disease-limiting vaccines, and transmission blocking vaccines that halt the spread of infection (Coelho *et al.*, 2017), there is an upside to the situation. Improvement and advances in post-

genomic technologies have facilitated the identification of numerous vaccine candidates, elucidating the gap in development of robust correlates of protection to adequately validate these candidates (Tuju *et al.*, 2017). Recent work could lead to the identification of a subset of VSAs for development of a severe disease prevention vaccine or a test to predict children at risk of severe disease children (Travassos *et al.*, 2018). Other available data could also make it possible to target antigens related to phenotypes such as rosetting (Vigan-Womas *et al.*, 2011) or to target parasites that prefer, and are linked to more severe disease syndromes (Storm *et al.*, 2019). Vaccines targeted at these antigens would prevent the specific severe disease manifestation of cytoadhesion and/or sequestration in malaria. More recently, studies comparing transcriptomes of parasites from severe and uncomplicated disease revealed both known and novel PfEMP1 linked to severe disease, and could greatly enhance the identification of severe disease vaccine targets (Tonkin-Hill *et al.*, 2018).

CHAPTER THREE

3.0 METHODS

3.1 Study location, subjects and recruitment

The study was conducted at the Hohoe Municipal Hospital in the Volta Region of Ghana, which is situated in the town of Hohoe (7.1519°N, 0.4738°E), with a population of about 63,000 inhabitants. The municipality is in the eastern central zone of Ghana, east of the Volta river and near the border to the Republic of Togo (Figure 3.1). The Hohoe district is located in the Wet Semi-equatorial climatic zone of the region, with perennial transmission of *P. falciparum* peaking seasonally in June-July during the April to November wet season. There is therefore an intense and prolonged malaria transmission season for the municipality. There are occasional reports of *P. ovale* and *P. malariae* infection, but by far the most dominant malaria species in the zone is *P. falciparum* (Kweku *et al.*, 2008). Annual precipitation values range between 1016 and 1210 mm, with a 4-5-month dry season between November and April. (Ghana Statistical Service, 2014). The 2010 national census puts the population of the municipality at 262,046, with 96,504 under 15 years-of-age (Ghana Statistical Service, 2012).

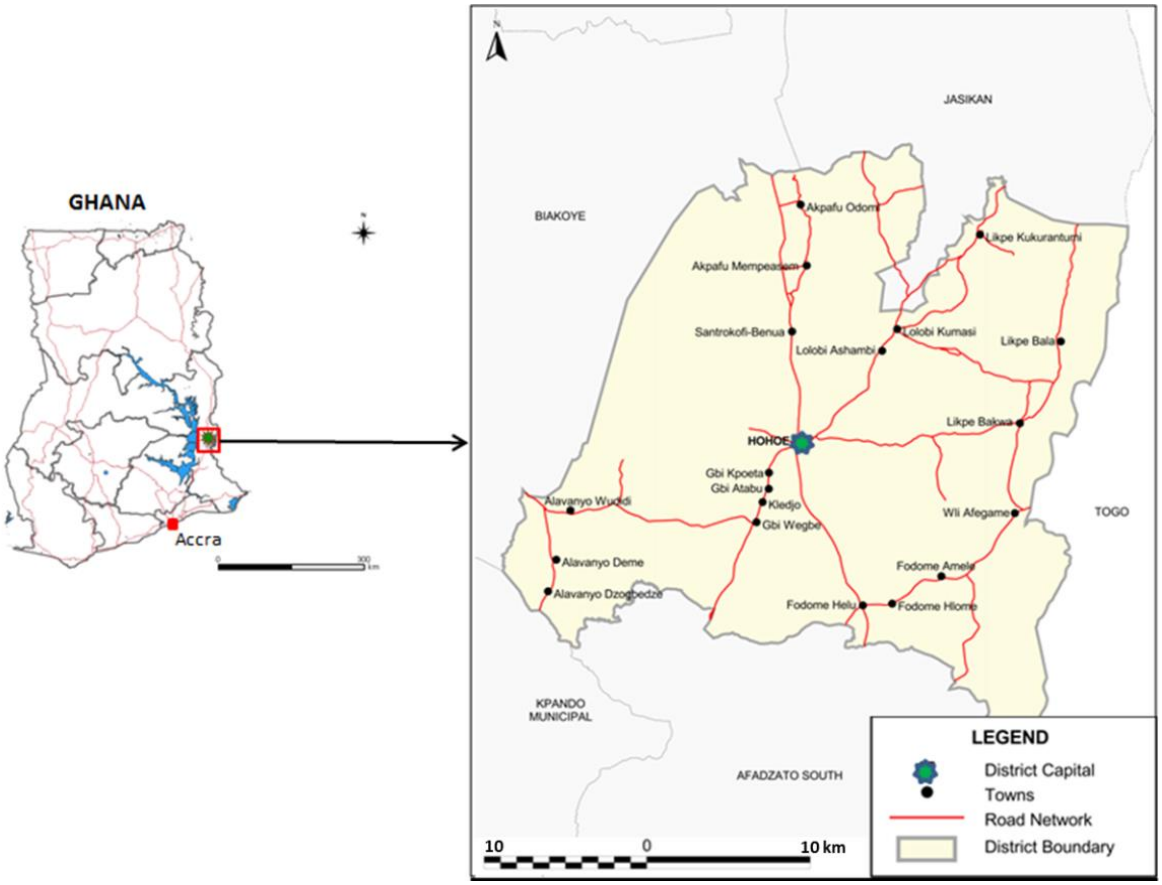


Figure 3.1 **The map of Ghana showing the field study site.** Hohoe is the district capital, located in the Hohoe Municipality of the Volta Region in eastern Ghana, about 221 km from the capital Accra. Map of the municipality adapted from the Hohoe District Analytical Report, Ghana Statistical Service (2014)

Permanently resident children aged 12 years and below from Hohoe and surrounding settlements who presented with malaria at the Hohoe Municipal Hospital were included in the project following written consent from their parents or guardians and satisfactory study requirements. Details of the study were described to each parent or guardian, including the option to exit the study after consent. Translation to the local language

during the consent seeking process was done where necessary. In addition, parents or guardians who did not sign the consent form, and children who did not meet the study requirements (due to diagnosis with a major medical condition such as cardiovascular disease), or those with SS sickling genotypes, were excluded.

Appropriate protocols and permits for the study were reviewed and granted by the Noguchi Memorial Institute for Medical Research Institutional Review Board (Study number 026/13-14), University of Ghana, and the Ghana Health Service Ethical Review Committee (GHS-ERC 08/05/14), Ministry of Health, Ghana.

The World Health Organization standard definitions and criteria for severe malaria and complications were employed in the collection and categorization of clinical and research blood samples (WHO, 2000b). Diagnosis involved a positive result from a *P. falciparum*-specific rapid test kit (First Response HRP2, India) and confirmation by microscopy of Giemsa-stained blood film. Patients with asexual *P. falciparum* parasitaemia of 2,500 IEs/µl of blood or more and temperature greater than 37.5°C, without an alternative cause of febrile illness were eligible for inclusion in the study. Recruited malaria patients were clinically examined by designated project clinicians and categorized into uncomplicated or severe cases following WHO guidelines on uncomplicated and severe malaria.

3.2 Blood sampling and processing

Rapid diagnostic testing for malaria was usually done as initial screening in the waiting area using finger-prick capillary blood, and the results confirmed by microscopy. For rapid test and/or microscopy positive children, venous blood samples were subsequently drawn by project staff for parasite counts (microscopy), *in vitro* cultures,

cryopreservation of parasite isolates, ABO blood grouping, blotting onto filter paper for subsequent parasite typing as well as for the tests described below. Fully automated blood chemistry and 18-parameter haematology indices were also performed. All patients were screened for HbS and G6PD deficiency.

Trained phlebotomists collected venous blood aseptically from the upper arm with a single use, sterile butterfly needle kit into evacuated 6ml EDTA and Heparin blood collection tubes (Becton Dickinson, USA) for molecular and cellular assays respectively. The vacuum tubes were centrifuged at 800 × g for 5 minutes to obtain the blood pellet and plasma, which was collected and stored in aliquots at -40°C.

For each patient, thick and thin blood smears were prepared on a microscope slide for estimation of parasitaemia according to WHO protocols (WHO/UNICEF, 2015b, WHO, 2010). Briefly, a small aliquot of blood was spotted near one edge of the slide and spread evenly but thickly in a circular motion to about 1.5cm in diameter. A second blood spot was then placed near the first and spread evenly along the length of the slide using the edge of a second slide to form a thin film. The slides were air dried, after which the thin smear was fixed by briefly flooding with, or dipping in 100% methanol. Once dry, the slides were stained for 15 minutes in a freshly prepared 10% Giemsa stain solution using Giemsa/staining buffer with a pH of 7.2. The stain was washed off by rinsing the slide under a gentle flow of water, air dried and examined under oil immersion with a light microscope (Olympus CX) at 1000× magnification. Parasitaemia was calculated from counts of *P. falciparum* infected RBC per 400 RBC when >1%, or else from counts of parasites per 200 WBC in the thick smear and adjusted to parasites per µl of blood using the measured RBC or WBC counts.

3.2.1 ABO blood grouping and Rhesus status

Blood grouping tests were carried out on all samples collected from patients using a commercially available ABO- and Rhesus-grouping kit (Fortress Diagnostics, UK) based on agglutination of RBC upon addition of anti-A, anti-B or anti-Rhesus reagents from the test kit. All reagents and samples were brought to room temperature for the test.

A drop of anti-A, anti-B and anti-Rh serum was placed on a clean, hard but smooth white surface, and to each of which a drop of blood from a patient donor was added. Each was mixed well with a small wooden stick and observed for agglutinating RBCs. A donor's blood type was categorized as blood type A if agglutination was observed with the anti-A serum only, or blood type B if the agglutination was observed with the anti-B serum only. Agglutination of RBC with both anti-A and anti-B test sera indicates the AB blood type, while no agglutination in both test sera is indicative of a donor of blood group O.

Similarly, an Rh-positive test of agglutinating RBCs following the addition of anti-Rh serum was used to determine if patients were Rhesus positive or negative.

3.3 Parasite cultures

P. falciparum field isolates obtained from patient blood draws were either cultured for use in rosetting assays or cryopreserved for later work. Blood stage culture procedures were all performed in a biosafety level II cabinet using standard methods (Cranmer *et al.*, 1997), with slight modification.

After collecting the plasma from the EDTA and heparin tubes as described in the previous section, the remaining RBC pellets were washed twice by centrifugation in excess, sterile, incomplete medium (RPMI 1640 medium with 25 mM Hepes, Sigma-

Aldrich, Germany, supplemented with 5µg/mL gentamicin sulphate) to dilute out any traces of anticoagulant. After washing, the medium was pipetted off and a 200 µl aliquot of the remaining pellet containing the IEs was added to a sterile 25 cm2 culture flask (Thermofisher Scientific, Denmark) containing 5mL of complete parasite medium (HEPES buffered RPMI 1640 medium supplemented with Albumax II (Life Technologies, Paisley UK), gentamycin and L-glutamine at 5, 0.05 and 0.18 mg/mL respectively and for field isolates, additional supplementation with 2% human serum) to obtain a final haematocrit of 4%. The flask was gassed with a mixture of 2.0% O_2, 5.5% CO_2 and 92.5% N_2 for 30 seconds, capped and incubated at 37°C, with regular media changes every 24 to 48 h, along with an estimation of parasitaemia from Giemsa-stained smears as described in the previous section to monitor the health and growth of *P. falciparum* in the cultures.

Maintenance of laboratory strains of *P. falciparum* used in this study, including 3D7, FMG and FUP followed the same procedure, but without supplementation of the complete media with human serum. Blood group O+ erythrocytes obtained from the blood bank (Rigshospitalet, Copenhagen, Denmark) were used for culturing of laboratory strains, after washing at least twice in incomplete medium to remove plasma and leucocytes. The washed RBC was re-suspended at 50% haematocrit, stored at 4°C and used for a maximum of three weeks. Genotyping of parasites in culture and tests for *Mycoplasma* contamination was carried out regularly every few weeks as described (Bengtsson *et al.*, 2013).

3.3.1 Freezing and thawing of parasites

3.3.1.1 Freezing P. falciparum-containing blood samples

P. falciparum-IE obtained from patients in the field during blood collection or from continuing cultures in the laboratory were prepared for long-term cold storage using the Glycerolyte method. In short, cultures with at least 2% (ideally 5%) ring forms were chosen for freezing. The cultures were initially transferred into sterile 12 mL tubes and centrifuged at 2000rpm (769 × g) for 5 minutes to pellet the RBC. The media/supernatant was removed and the pellet re-suspended and its volume estimated. The total volume of glycerolyte to be used was determined from the pellet volume, V, by multiplying V by a factor of 5/3. Next, one-fifth of V (glycerolyte) was added dropwise to the pellet using a 2 mL syringe and an appropriate sized needle. The mixture was incubated for 5 minutes at room temperature followed by the addition of the rest of the glycerolyte (the remaining 4/5 × V), also dropwise, while gently shaking the tube. The mixture was then transferred into cryotubes at a volume of 800 µl each, placed into a 'Mr Frosty' freezing container (Thermo Scientific) and stored at -80°C overnight before they were transferred to an ultra-low -150°C freezer or into liquid nitrogen.

3.3.1.2 Thawing P. falciparum-containing blood samples

Malaria parasites were thawed from -150°C or liquid nitrogen storage using two sterile NaCl solutions (12% and 1.6%) at room temperature. In brief, the vial to be thawed was removed from cold storage, quickly thawed in a water bath at 37°C and transferred aseptically into a 50 mL centrifuge tube. The volume of thawed suspension, V, was noted, following which 0.1 × V of the 12% NaCl solution was added while gently shaking the tube. The tube was left for 5 minutes, after which 10 × V of 1.6% NaCl was

also added dropwise as before. The tube was centrifuged at 20°C for 5 minutes at 500 × g, after which the supernatant was discarded and the pellet was resuspended in 10 × V of culture medium and centrifuged at 500 × g under the same conditions. Following this step, the pellet was re-suspended in the appropriate amount of culture medium, transferred to a culture flask, gassed and placed in a 37°C incubator.

3.4 Detection of rosetting rates in selected parasites and field isolates

Rosetting assays were used to measure the rosetting rates in blood group antigen selected *P. falciparum* laboratory strains, and also in parasite isolates from patients. In the field, rosetting assays were performed following parasite culture in the donor's own RBC for 24-36 hours when the parasites were at late trophozoite/early schizont stages.

For laboratory strains (3D7, FMG, HB3 and FUP), the selected parasites were cultured in blood type O RBC using standard protocols as described in Section 3.3. Mature parasites to be used for rosetting assays were concentrated, as described in detail in Section 3.8.1, by magnetic-activated cell sorting (MACS) and mixed with uninfected RBC of blood type O, A or AB for 1 h prior to counting the rosettes.

For the rosetting assay ethidium bromide was added (at a final concentration of approximately 7 µg/mL) to a 100 µL aliquot taken from a thoroughly but gently mixed late stage culture, incubated for at least 2 minutes at 37°C after which the samples were ready for observation. Two to 5 µL of the mixture was then transferred by micropipette to a well of an 8-well 6 mm diagnostic microscope slide (ThermoFisher Scientific, USA) and gently covered with a coverslip to avoid trapping bubbles underneath. The RBCs and rosettes were observed using a fluorescence microscope at 400×

magnification. The rosetting rates were assessed by detection and counting the number of rosettes observed per 200 IEs and expressed as a percentage.

3.5 Blood group polysaccharide constructs

Four bovine serum albumin (BSA)-coupled blood group neoglycoprotein (NGP) constructs were purchased as lyophilized powders from Dextra Laboratories Limited, Reading, UK (https://dextrauk.com/) for use in the parasite selection and binding assay experiments. These NGPs, namely Blood Group A-BSA (6-atom spacer), Blood Group B-BSA (6-atom spacer), Blood Group B-BSA (3-atom spacer) and Blood Group H disaccharide-BSA (3-atom spacer) (product codes NGP6305, NGP6323, NGP0323 and NGP0205 respectively) were reconstituted to a stock concentration of 250 µg/mL with sterile PBS. They were further diluted to a final concentration of 10 µg/mL for the experiments.

3.6 Selection for binding to blood group constructs

The 3D7, FMG, HB3 and FUP parasite strains were selected on blood group polysaccharides and used for subsequent assays. All procedures were performed under sterile conditions, and all media pre-warmed to 25-37°C.

A day before selection, one well of a 24-well plate (Nunc, Thermo scientific) was coated overnight at 4°C with 400 µL of 10 µg/mL oligosaccharide-BSA in PBS. On the day of selection, the buffer was aspirated and the well was blocked for 2 hours at room temperature with 800 µl of 2% BSA in PBS. The blocked well was then washed 3 times with 1 mL of culture medium each time.

Late trophozoites and schizont stages of the culture to be used for selection were purified by MACS as per the manufacturer's instructions (Section 3.8.1) under sterile conditions, using PBS with 2% FCS, RBC-wash or culture medium.

The IEs were re-suspended in a final volume of 400 µl in culture medium, transferred to the coated well and incubated with gentle shaking for an hour at room temperature. Following incubation, repeated gentle washing to remove unbound IEs was done by first adding, and then aspirating 1 mL of culture medium (the well was not emptied before first wash). The washing procedure was repeated 3 to 7 times depending on how well parasites bound. An inverted microscope was used to check bound cells after every 2-3 washes.

After washing, between 200 and 400 µl of culture medium and 50 µl of packed RBC were added to the excess medium in the wells to ensure a minimum volume of 600 µl per well. The plate was incubated in an exicator under micro-aerobic conditions at 37°C overnight. The following day, 500 µl of culture medium was carefully removed from the top, leaving the RBCs at the bottom. A thin smear was prepared from this to observe selected parasites that were now at ring stages, before the remaining RBC were transferred to a culture flask, adjusting for the appropriate haematocrit for the volume used.

3.7 Blood group antigen and EC binding assays

3.7.1 ABO polysaccharide antigen binding assay

Parasites selected on blood group A or B polysaccharide constructs, along with field isolates cryopreserved from patients from the field site were tested for binding to the blood group antigens in a modification of the method described by Hempel and colleagues for immobilized cells (Hempel *et al.*, 2015).

The wells of flat-bottomed optical 96-well polystyrene plates (Nunc Polysorb, Thermo Fisher Scientific) were coated overnight at 4°C with 50 µL of blood group A-, B- or H-BSA constructs diluted to a concentration of 10 µg/mL in 0.05 M Carbonate buffer, pH 9. Following the coating step, the excess constructs were flipped out and the wells were blocked with 100 µL/well of 20 mg/mL (2%) BSA in PBS for a minimum of 2 hours at room temperature. The wells were washed twice with 100 µL complete parasite medium, followed by the addition of 100 µL of the late stages of control (unselected) and IE of selected and field isolates to the wells at a concentration of 1.25×10^7 cells/mL (or 1×10^6 cells/100 µL). The plate was incubated with gentle shaking at room temperature for 45-60 minutes. A gravity wash step to remove unbound IE was done by immersion and inversion of the plate in 2% FCS in PBS for 60 to 90 minutes, after which it was taken out and blotted to remove the excess wash buffer. Standards (known concentration/quantity of RBC or IE) were added to the plate and incubated for 15 minutes with 100 µL of 0.1% Triton X-100 (Sigma-Aldrich) diluted in PBS to permeabilize the bound erythrocytes in the wells. This disruption of IE enables the release of intra-erythrocytic haem which acts as a quantifiable pseudoperoxidase (Hempel *et al.*, 2015). Tetramethylbenzidine (TMB, R&D Systems, Oxon UK) was added to the wells at 100 µL/well, and after incubation in the dark for about 5 minutes

while monitoring colour development, the reaction was stopped by the addition 50 µL of 1M H_2SO_4 to the wells. Optical densities of the wells were read at a wavelength of 450 nm with a 540 reference on a plate reader (Multiskan EX, Thermo Labsystems, MA, USA).

The proportion of adherent IEs were determined by the formula:

$$\frac{(\text{sampleOD} - \text{backgroundOD})}{(\text{totalOD} - \text{backgroundOD})}$$

where backgroundOD = OD of wells with no added IE, and totalOD = OD of well with total number (100%) of IE (1×10^6 RBC or IE).

3.7.2 Endothelial cell binding assays

Primary human endothelial cells of the aorta and foreskin dermis were cultured *in vitro* and used in cell binding assays to assess the binding characteristics of blood group A- and B-binding 3D7, HB3 and FMG parasites. The assays were performed using 96-well flat-bottomed tissue culture plates (NUNC, Denmark) containing monolayers of the relevant cell lines. Briefly, and generally as described in the method by Hempel *et al.* (2015), ECs were seeded in the wells at approximately 1.5×10^5 cells/ml and grown to confluence 48 hours prior to the experiment. On the day of the experiment, MACS-purified late stages of the parasites (Section 3.8.1) were added in triplicate to the EC-containing wells at a concentration of 1×10^6 IE in 100 µL per well (about 1.25×10^7 cells/mL). Subsequent steps were essentially as described for the blood group binding assay described in the previous section. Thus, the plate was incubated with gentle shaking for about 45- minutes to 1 hour at room temperature and then gravity-washed in 2% FCS in PBS for 60 to 90 minutes to remove unbound erythrocytes. After blotting on

tissue paper to remove excess wash buffer, the permeabilization step followed with the addition of 100 µL of PBS supplemented with 0.1% Triton X-100 to the wells. After 15 minutes, TMB was added to the wells at 100 µL/well, and colour development was allowed to proceed in the dark. The reaction was stopped with 50 µL of 1M H_2SO_4/well and ODs read at 450nm in an ELISA plate reader. Similarly lysed IE of known concentration were included on the plate, as well as wells with EC but with no added IE to serve as standards and background references respectively. The percentage of adherent cells were determined using the formula

$$\frac{(\text{sampleOD} - \text{backgroundOD})}{(\text{totalOD} - \text{backgroundOD})}$$

as described in the preceding section (3.7.1).

3.7.3 Blocking assay using anti-A and anti-B monoclonal antibodies

A variation of the binding assays described previously was conducted using commercially procured monoclonal antibodies to blood group A and B antigens. The antibodies were used to confirm the uniqueness and difference between the blood group constructs by specifically blocking adhesion of IE of selected parasites to the respective polysaccharide constructs A-BSA and B-BSA used in this study. As described earlier in Section 3.7.1, 96-well microtiter plates were coated overnight with the blood group A-, B- and H-BSA constructs. The plates were blocked as before for 2 hours with 2% BSA in PBS and washed four times with 100 µL complete parasite medium. After the washing step, mouse Monoclonal Anti-Blood Group A and Monoclonal Anti-Blood Group B antibody (SAB4700677 and SAB4700676 respectively, Sigma Aldrich) were diluted in PBS and incubated at 100 µL per well for 1 hour. The washing step was repeated after the incubation period, followed by the addition of horseradish peroxidase

(HRP)-conjugated Goat anti-mouse IgM secondary antibody (Jackson ImmunoResearch 115-035-020 from TriChem, Denmark) diluted 1:5000 in PBS, 100 µL/well for an hour. After another washing step, TMB substrate was added and the colour allowed to develop prior to reading the plate in an ELISA plate reader to obtain the ODs as before. All steps except for the coating were performed at room temperature.

3.8 MACS and Flow Cytometry

3.8.1 Harvesting late stage *P. falciparum* by Magnetic-activated cell sorting

Late trophozoite stages of *P. falciparum* were harvested from culture by MACS (Miltenyi Biotec, Germany) following the manufacturer's instructions. Briefly, the column and 3-way stopcock were assembled in the magnet and prepared by attaching a 20 mL syringe filled with PBS containing 2% FCS (PBS-2) to the left opening of the stopcock. The tap was set to a position to open the way between the syringe and column but close the way out. The plunger was moved back and forth until the column was well rinsed and all bubbles were dislodged from the column. A blunt needle (20G) was attached to the lower outlet of the 3-way stopcock, which was opened between the column and the lower outlet to allow the PBS-2 to run through to a waste receptacle below. A further 20 mL of PBS-2 was added gradually to the top of the column and allowed to run through, making sure the column did not run dry so as to avoid air pockets. The culture to be separated was then re-suspended with 5 mL of PBS-2 and added to the column, followed by 40-50 mL more PBS-2 to rinse out excess, unattached ring-stage IEs and uninfected RBC. A plunger attachment was inserted at the top of the column, to which a syringe filled with 20 mL of PBS-2 was fitted. The column with the attached syringe was twisted to unscrew it from the 3-way stopcock, detached from the magnet and inserted into a 50mL tube. The IE containing the late stage parasites trapped

in the column were removed by pushing the PBS-2 in the attached syringe through the column into the 50 mL tube. The late stage parasite concentration in the resulting suspension was estimated by counting the IE using an Improved Neubauer haemocytometer.

3.8.2 Measurement of antibody reactivity to selected parasite VSA by Flow Cytometry

Antibody reactivity to variant surface antigens of parasites selected on blood group antigens was investigated by flow cytometry. Plasma samples from children enrolled in the study and stored at minus 40°C were thawed and added to 96-well polystyrene round-bottom microtiter plates (Nunc, Thermo Fisher Scientific) at 5µL per well. IEs containing late stage parasites obtained from MACS separation as described in Section 3.8.1 were then adjusted to a concentration of $2x10^6$ cells/mL in 2% FCS in PBS and added to the well at 100 µL per well. The plate was then incubated for 30 minutes at 4°C, after which the cells were washed twice in PBS-2 by centrifugation at 500 × g for 4 minutes each time. The wash buffer was removed after each wash by flicking the plate once, leaving the IE pellets at the bottom of the wells. Next, 100 µL of FITC conjugated Rabbit anti-Human IgG antibody (Jackson ImmunoResearch) was diluted 1:150 in PBS-2 with 20 µL/mL of 0.1 mg/mL Ethidium Bromide solution (2 µg/mL) and incubated again for 30 minutes at 4°C. The cells were then washed twice as before, followed by the addition of 100 µL of PBS-2, after which the plate was read with a flow cytometer (Cytomics FC500, Beckman Coulter, USA). WinList version 6.0 (Verity Software House Inc.) was used to analyze the data generated. Uninfected and IE were gated according to ethidium bromide fluorescence.

3.9 Molecular methods

3.9.1 Sample processing and storage

Laboratory isolates, both unselected and selected for binding to blood group oligosaccharides (Section 3.6), were processed and stored in TRIzol from cultures at ring stages (at least 2%) by transferring the contents of the culture flasks to centrifuge tubes and pelleting IE by centrifugation at 500 × g for 5 minutes. Following centrifugation, and removal of the supernatant/medium, 200 µL of the RBC pellet containing the parasites was pipetted into a cryo-storage tube, to which 1.6mL of TRIzol (ThermoFisher Scientific InVitrogen, USA) at room temperature was added. This was thoroughly mixed by pipetting several times to remove clumps and maintain parasite RNA integrity. After a brief shake in the vortex, the tubes were transferred to a -80°C storage facility until use.

3.9.2 RNA extraction from Trizol-stored samples

Samples stored in TRIzol and retrieved from -80°C storage were thawed to room temperature, 500 µL of which was transferred into an Eppendorf tube after pipetting up and down a few times for a uniform mixture. To this tube 200 µL of chloroform (Sigma-Aldrich, Germany) was added and shaken vigorously for about 15 seconds to achieve a good mix and then centrifuged at 12,000 × g for 15 minutes at 4°C. The supernatant was collected and transferred to a new Eppendorf tube, after which 500 µL of 2-Propanol (Sigma-Aldrich, Germany) and 5 µL of Glycogen (ThermoFisher Scientific, USA) were added. The mixture was shaken and then incubated for 10 minutes at room temperature, before spinning at 12,000 × g for 10 minutes at 4°C in the centrifuge. The supernatant was removed, and the remaining pellet was washed by centrifugation (5 minutes at 7,500 × g at 4°C) by adding 1mL of 75% Ethanol in 25%

DEPC (diethylpyrocarbonate) water (deionized) to the pellet. The supernatant was then carefully pipetted off and the pellet air dried for a few minutes, before adding 10 µL of DEPC water and placing the tube in a heat block at 65°C for 5 minutes to dissolve the pellet. The extract was then transferred onto ice or stored in the freezer for the next step. All steps described in this extraction procedure were performed in a fume hood, and using RNase-free tips and consumables to minimize contamination.

3.9.3 DNase treatment and cDNA synthesis

Following RNA extraction, samples were treated with DNase to digest possible genomic DNA contaminants. Briefly, 10 µL of the extracted RNA was transferred to a PCR tube and incubated for 20 minutes at 37°C with 1.4µL of 10× DNase reaction buffer (Sigma-Aldrich, Germany) and 2µL of DNase I (Sigma-Aldrich, Germany). At the end of the incubation period, 2µL of stop solution (Sigma-Aldrich, Germany) was added to the mixture and kept for 10 minutes at 70°C to stop the reaction.

To confirm presence of extracted RNA and the effectiveness of DNase treatment of the sample, an aliquot of the DNase treated RNA sample was tested by QPCR using a primer set for the p90 *seryl-tRNA synthetase* housekeeping gene (Salanti *et al.*, 2003).

In brief, 0.5 µL of the treated RNA sample was added to 10 µL of SYBR Green PCR Master Mix (Qiagen, UK), 7.5 µL DEPC water and 2 µM forward and reverse primer pair of the p90 housekeeping gene. The QPCR was performed on a Rotorgene RG-3000 thermal cycler (Corbett Research, Australia).

Complementary DNA (cDNA) synthesis from the DNA-free RNA was achieved by reverse transcription from random hexamers using Superscript II (InVitrogen, USA), as described in the manufacturer's protocol. In brief, to a 200 µL PCR tube was added 15 µL of the RNA sample, 0.75 µL of random primers (3 µg/µL in RNase-free

water)(ThermoFisher Scientific, USA) and 1.5 µL of 1.74 µM Deoxynucleotide triphosphate (dNTP) mix (InVitrogen, USA). The tube was then vortexed, spun briefly, incubated for 10 minutes at 65°C, and then transferred directly onto ice. Six microliters of First Strand Buffer (ThermoFisher Scientific, USA), 3 µL of dithiothreitol (DTT) (ThermoFisher Scientific, USA), and 1 µL each of RNaseOut and Superscript II (ThermoFisher Scientific, USA) were added to the reaction tube, which was then vortexed to mix the contents. Following a brief spin, the tube was placed in a PCR machine with the following settings for cDNA synthesis: 1 cycle each of 25°C for 10 minutes, 42°C for 50 minutes, 70°C for 15 minutes, and then 8°C.

cDNA samples were again tested for gDNA contamination by QPCR with p90 primers as described earlier in this section.

3.9.4 Quantitative Polymerase Chain Reaction to detect VSA transcripts of *var* and *rif* genes

Complementary DNA samples from blood group selected parasites processed as described in section 3.9.1, and free from gDNA contaminants, were prepared for reverse transcriptase (RT)-PCR on a thermal cycler (Rotorgene RG-3000, Corbett Research, Australia). The reactions were carried out in a total reaction volume of 20 µL, in 0.1 mL clear polypropylene tube and cap strips containing 10 µL QuantiTect SYBR Green PCR master mix (Qiagen, UK), 2 µM forward and reverse gene primer pairs, and 0.2 µL cDNA sample. Primer pairs of 154 *rif*, 60 *var*, and primers to the endogenous control genes *seryl-tRNA synthetase* (p90) and *fructose-bisphosphate aldolase* (p61), were used (Wang *et al.*, 2010, Dahlback *et al.*, 2007).

Optimised cycling conditions for cDNA of *P falciparum* were as described previously (Salanti *et al.*, 2003) as follows: 95°C for 15 minutes, followed by 40 cycles of 95°C for 30 s, 54°C for 40 s and 68°C for 50 s.

3.9.5 Statistical Analysis

Statistical analysis of the data generated in this study were carried out using Sigmaplot 12 (Systat Software, Inc., San Jose, CA) and GraphPad Prism (Version 6, GraphPad Software, San Diego, CA). Characteristics of study participants were recorded as median with minimum and maximum values. As most of the data were not normally distributed, the non-parametric Mann-Whitney and Kruskal Wallis tests were used to determine differences between two groups, or in comparing more two groups, respectively. Spearman's Rank-Order coefficients were also calculated to assess correlations, and in cases where post hoc tests were required following multiple comparisons, the Dunn's Multiple Comparison test was employed. P values ≤ 0.05 were considered statistically significant.

CHAPTER FOUR

4.0 RESULTS

The initial aspect of this study was to establish blood group A and B-binding variants of well-characterized *P. falciparum* laboratory strains and to demonstrate the resulting phenotypic changes. *P. falciparum* 3D7, FMG and FUP isolates were continuously panned on blood group oligosaccharides immobilized on polystyrene plates to yield blood group A and B binding phenotypes. The effect of this selection of parasites on rosetting was then investigated by conducting rosetting assays of IEs containing selected parasites and RBCs from donors of blood types A, AB and O.

A plate-based quantitative read-out specifically measuring selected parasite-IE binding to immobilized A- and B oligosaccharide binding was therefore developed to measure this interaction. Initial development of the assay involved confirmation of binding of the BSA-conjugated oligosaccharide constructs to the polystyrene plates. This was achieved by detection of the bound constructs at different concentrations using blood group A, B and O specific biotinylated lectins as indicated in Table 4.1.

Ulex europaeus lectin (UEA) binding to the H-BSA construct was measured as the optical density (OD) and was concentration dependent. Additionally the *Galanthus nivalis* lectin, or GNL, used here as a negative control lectin, showed no indication of binding to the targets of interest (Figure 4.1a). Similarly, *Dolichos biflorus* agglutinin (DBA), which specifically binds the blood group A oligosaccharide, showed specific and concentration dependent binding to the immobilised blood group A construct (Figure 4.1b).

Table 4.1 Lectins and corresponding specific antigen/carbohydrates

Lectin	Blood Group Specificity
Dolichos biflorus agglutinin (DBA)	Anti-A1
Griffonia (or *Bandeiraea*) *simplicifolia* lectin (GSL-I or BSL-I)	Anti-B
Ulex europaeus agglutinin (UEA-I)	Anti-H (O)
Galanthus nivalis lectin (GNL)	Anti-rabbit RBC (does not bind A,B or H)

The DBA, GSL-I and UEA-I lectins bind specifically to blood group A, blood group B and blood group O antigens respectively. GNL on the other hand, is known to agglutinate rabbit, but not human erythrocytes

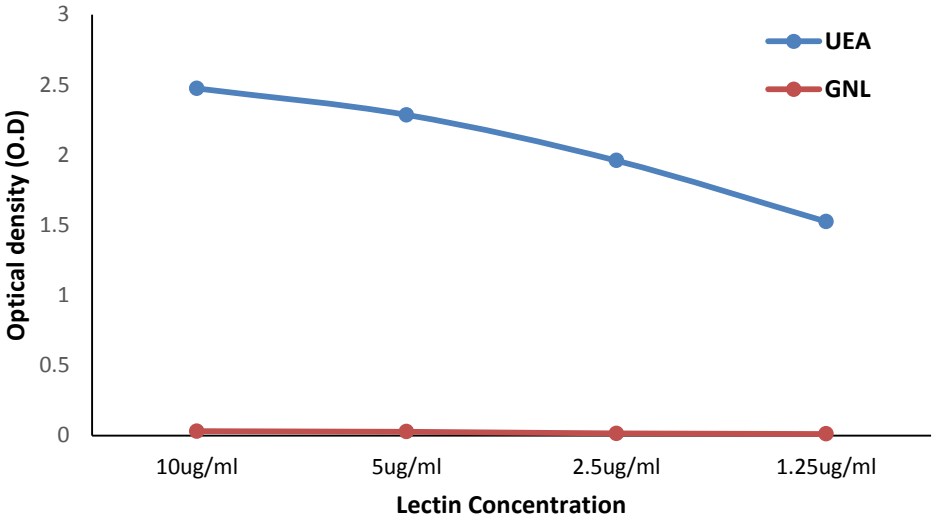


Figure 4.1a **Dose dependent binding of blood group O specific *Ulex europaeus* agglutinin (UEA) to blood group H antigen construct**. The H-BSA construct was coated at 10 µg/ml. Each data point is the mean of triplicate readings in an experiment. No OD signal was observed with the non-specific *Galanthus nivalis* lectin (GNL) at any concentration.

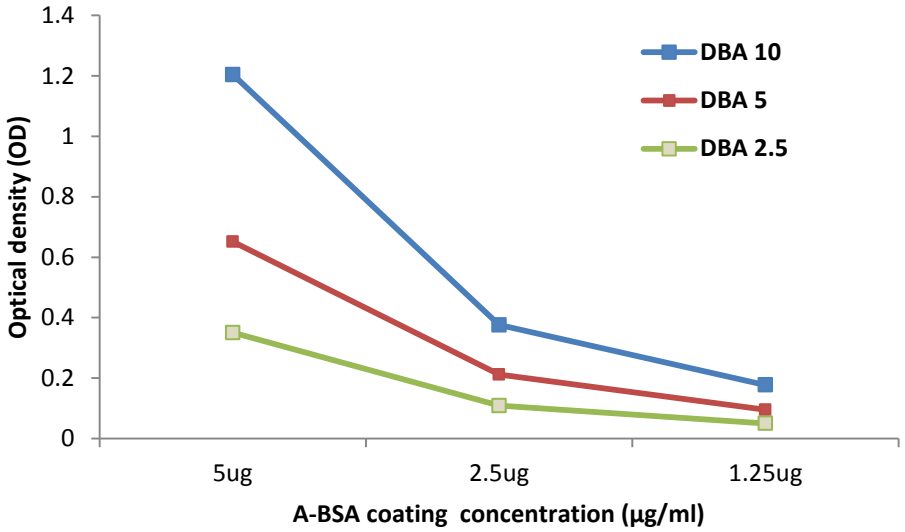


Figure 4.1b **Detection of immobilized A-BSA construct with *Dolichos biflorus* (DBA) lectin**. ODs show concentration dependent binding of the DBA lectin to the blood group A oligosaccharide construct coated at different concentrations. Each data point indicates mean of triplicate readings in an experiment

4.1 Rosetting in parasites selected on blood group antigens

To investigate the effect of selecting blood group binding phenotypes on rosette formation, parasites (3D7, FMG and FUP) selected by regular panning on BSA-coupled blood group oligosaccharides, along with their corresponding isogenic unselected cultures, were tested for rosetting using blood type A, AB and O RBCs from donors without malaria. Rosetting rates are the number of rosettes counted in ethidium bromide stained parasites per 200 IEs under a fluorescent microscope (Figure 4.2).

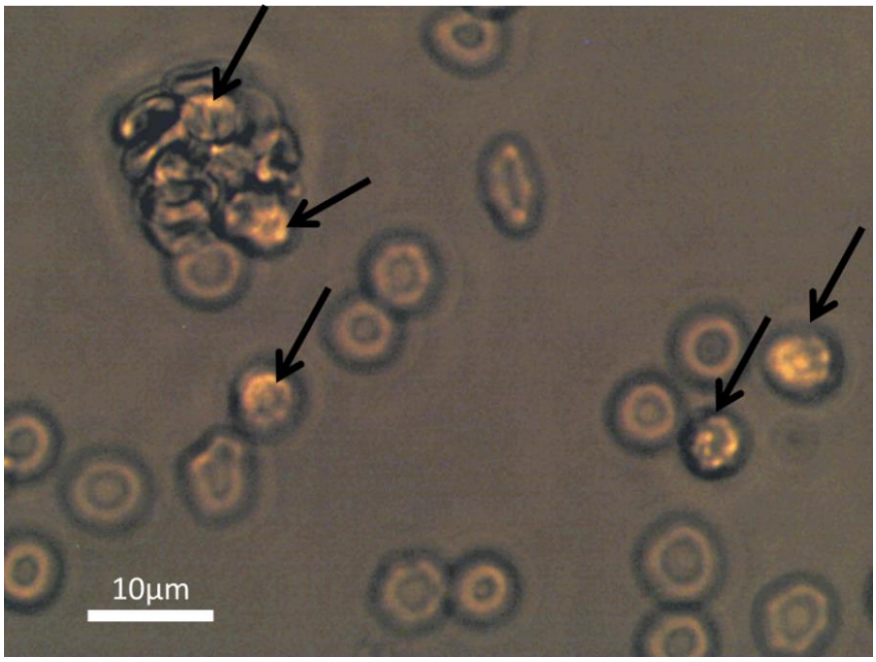


Figure 4.2 **A large rosette containing IEs surrounded by several RBC**. Ethidium bromide-stained late stage *P. falciparum* parasites can be seen in the rosette and in surrounding IEs (arrowed)

All parasites selected on blood group sugars clearly formed rosettes, while none were observed in the unselected 3D7, FMG and FUP parasites (Figure 4.3). It was observed particularly that FMG B-selected IEs in group A and AB RBC formed numerous giant rosettes. In addition, the rosetting rates observed were generally higher in group A

RBCs than rates observed in blood group AB erythrocytes. Irrespective of the parasite isolate or whether selected or unselected, no rosetting was observed in blood type O erythrocytes (Figure 4.3).

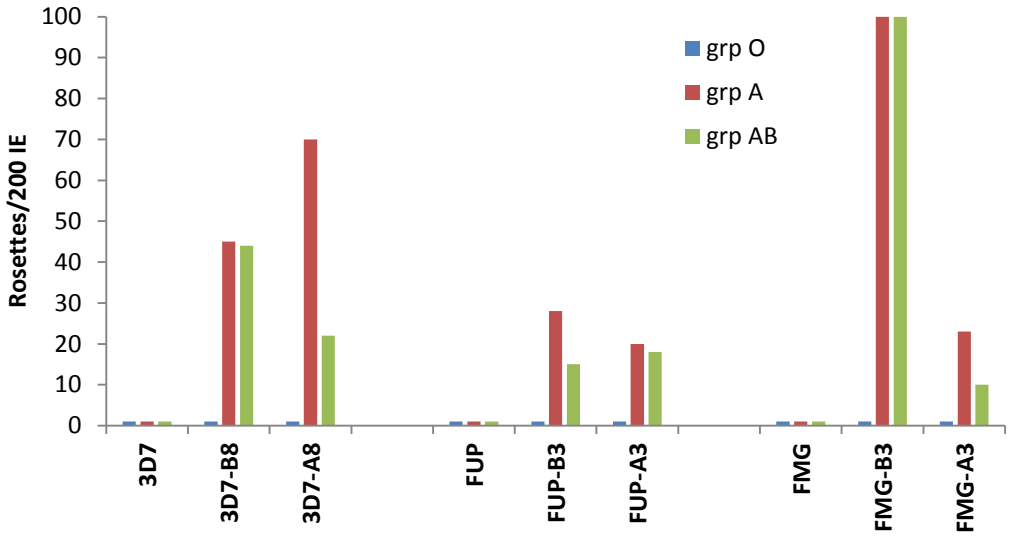


Figure 4.3 **Rosetting rates of selected parasites in RBC of blood group O, A and AB**. Unselected parasites (3D7, FUP and FMG) do not form rosettes in RBC of blood group O, A or B. However, A- and B-selected parasites (A3, A8 and B3, B8) all show increased rosetting rates in blood group A and group AB, but not in group O RBCs. Rosetting rates are the average of counts from duplicate wells for each isolate.

4.2 Adhesion of selected parasites to blood group oligosaccharides

With the culture of blood group polysaccharide-binding phenotypes, the plate-based assay described earlier at the start of this chapter was developed further and used to directly measure adhesion of IEs to immobilized blood group antigens.

Following three rounds of selection by panning on blood group A and B constructs, 3D7 parasites selected by this method consistently bound to A- and B-BSA constructs at much higher levels compared to the levels observed for the unselected parent culture. Binding to the group O antigen construct (H-BSA) by the selected parasites was low, and comparable to binding of the selected parasites (Figure 4.4).

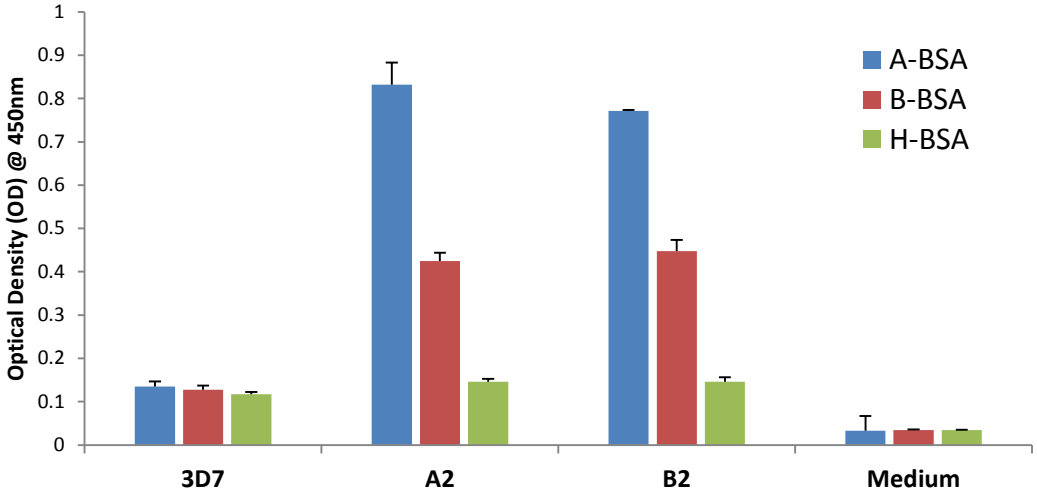


Figure 4.4 **Optical density readings of bound RBC in plate-based assay.** Typical binding profile of selected (A2, B2) and unselected 3D7 parasites to blood group constructs (A-BSA, B-BSA and H-BSA) following 3 rounds of selection by panning. Error bars denote standard deviation (SD) of duplicate readings from the same experiment.

To confirm the presence and availability of epitopes and to test the specificity of the blood group constructs used in the adhesion assays, monoclonal antibodies against blood group A and B polysaccharides were used. Panels A and B of Figure 4.5 show

clear, specific binding of the respective specific antibody to the A-BSA and B-BSA constructs. On the other hand, the monoclonal antibodies against blood group A do not recognize or bind B-BSA and vice versa.

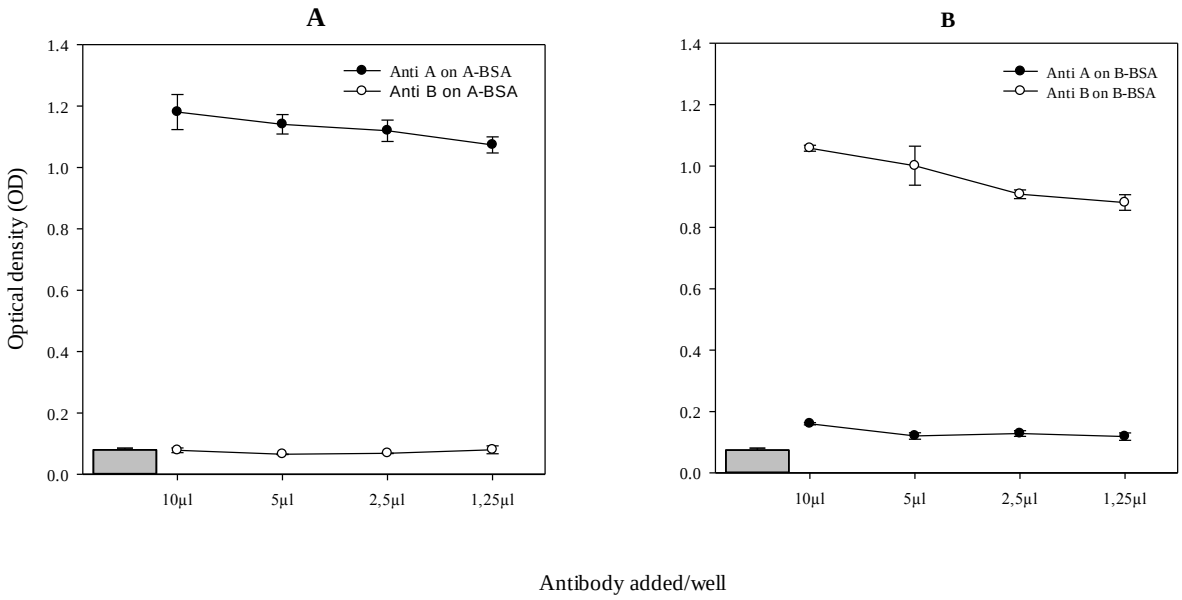


Figure 4.5 **Reactivity of mouse monoclonal anti blood group A and B antibody with A-BSA and B-BSA constructs by ELISA**. Panel A shows ODs of reactivity with blood group A-BSA construct, panel B shows reactivity with the B-BSA construct. The grey bars show OD of secondary antibody alone. Error bars are SD of duplicate readings

Additionally, it was demonstrated that these monoclonal antibodies to the blood group constructs could inhibit the specific binding of IE of selected parasites to the constructs (Figure 4.6). With no inhibiting antibodies added, the adhesion to both A-BSA and B-BSA constructs remained high at 60% and 58.5% respectively (White bars). This

dropped to 1% with the addition of anti A antibody and to 15.6% when anti B antibody was added. The addition of anti B antibody appeared to have no effect on the binding of the parasite isolate (B-selected) to A-BSA (Grey bar), while the anti A antibody reduced the binding to the B-BSA to 43.8%. Adhesion to the H-BSA construct with no added antibody was 6%.

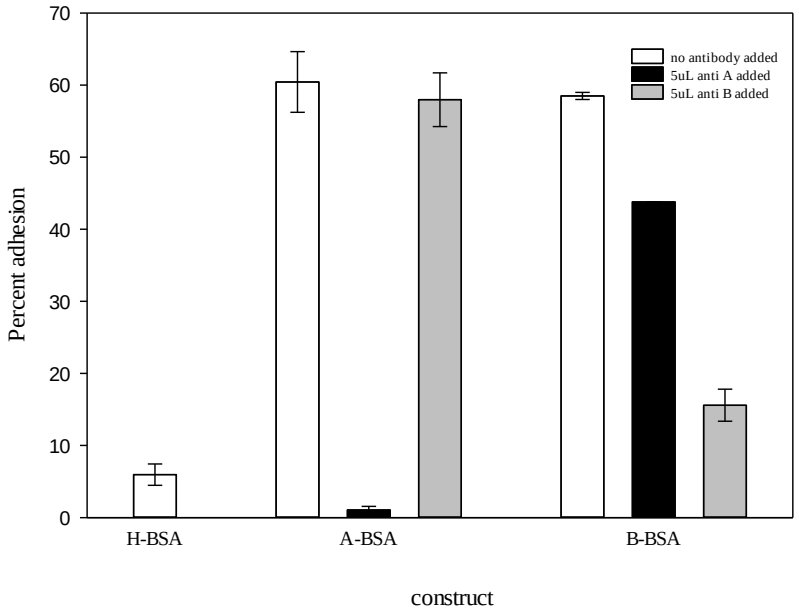


Figure 4.6 **Inhibition of blood group B selected parasite adhesion to constructs by specific anti A- and B antibody**. White bars represent adhesion to constructs with no antibody added, Black bars: adhesion with 5µL anti A antibody added, Grey bars: adhesion with 5µL of anti B antibody added. Error bars show SD of multiple readings

4.2.1 Characteristics of binding isolates

Binding of parasites to the H antigen was no different between 3D7, A-BSA selected or B-BSA selected parasites, and as expected, the unselected 3D7 parasites also showed no preference for binding to any of the 3 blood group antigens.

A-BSA selected parasites bound to blood group A polysaccharides 7 to 8 times more than unselected 3D7 parasites. The binding of parasites selected on B-BSA to the group B construct was usually slightly less, at 2 to 4 times more than the binding observed in the unselected 3D7 (Figure 4.7).

It was noted that selection of *P. falciparum* laboratory strains on either A or B blood group antigens always resulted in the selected parasites binding to both A and B constructs (Figure 4.4 and 4.7). In addition, the selected isolates bound better to the blood group A antigen than to the B antigen, with the binding of selected parasites to the blood group A polysaccharide usually about 1.5 times more than the binding to the blood group B polysaccharide.

Binding of all parasite isolates to the H antigen was observed to be quite weak, and attempts to select for binding to this construct following two rounds of selection yielded no clear result (Figure 4.7).

The H antigen construct was supplied with a 3 atom spacer arm, while the A and B constructs were linked with a 6 atom spacer to BSA (Section 3.5). To determine whether the lack of binding to the H antigen was due to the shorter spacer arm of this construct, binding to a B construct with the same shorter arm was tested. As Figure 4.8 shows, A and B selected parasites of 3D7 and FMG bound to both constructs (B3 and B6), albeit with consistently lower binding to the B-construct with the shorter spacer

(32-68%). Interestingly, selected FMG parasites on the other hand bound both B constructs equally well (100-105%). These results indicate that the shorter spacer arm of the H construct does not explain the lack of binding seen.

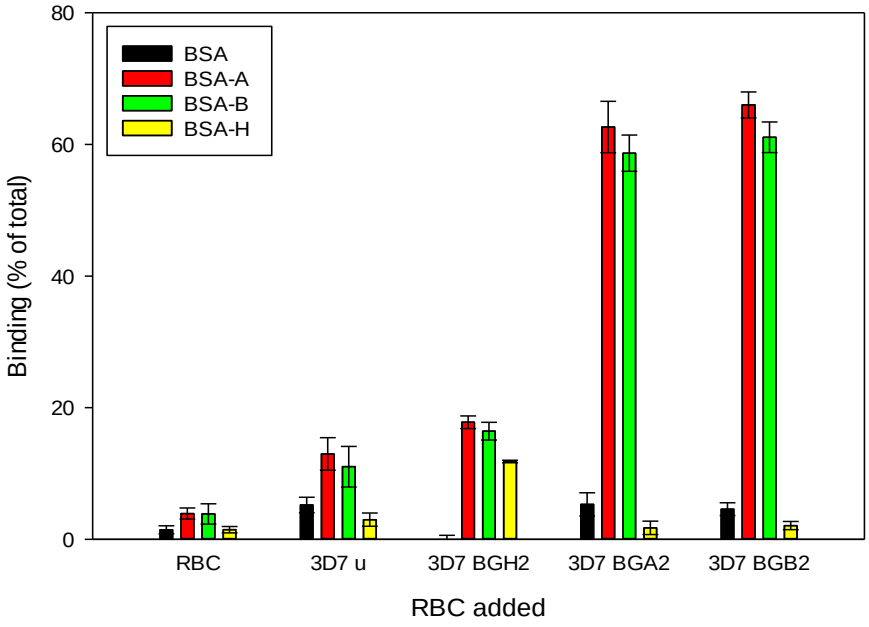


Figure 4.7 **Weak binding to the H-BSA (blood group O) construct by all parasites.** Percentage binding of parasites selected after 2 rounds of panning on H-BSA, A-BSA, and B-BSA constructs (BGH2, BGA2 and BGB2 respectively). Low level binding of IE to H-BSA was observed for all selected parasites (yellow bars). BGH2 parasites (3rd group of columns), showed no preference for binding to any of the constructs, even for H-BSA (less than 15%). Error bars denote standard deviation of duplicate measurements.

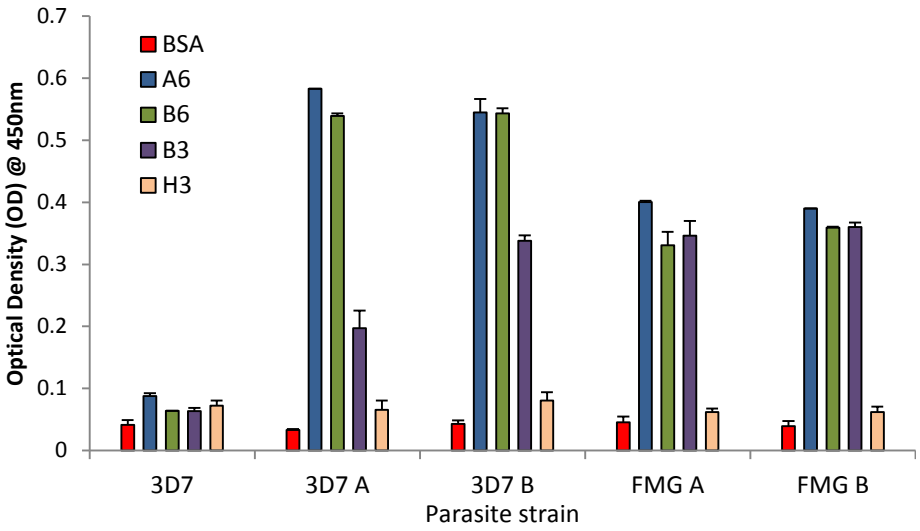


Figure 4.8 **A-BSA and B-BSA selected parasites of 3D7 and FMG bind both the 3- and 6-spacer arm constructs of blood group B.** Selected parasites 3D7 A and 3D7 B show better binding to the 6-spacer arm construct of B-BSA, B6 (green bars) than to the 3-spacer arm construct B3 (purple bars). Error bars represent standard deviation of duplicate readings

Subsequently, to investigate if phenotypic changes caused by selection on A or B antigen are a general phenomenon or limited to some strains, the experiments were repeated using other established laboratory strains of *P. falciparum*: FCR3, FMG, FUP and HB3. As observed with the 3D7 strain, selection of the FMG and FUP strains on the blood type sugars was successful and resulted in binding that was several times higher than for the corresponding unselected parental parasites. In contrast, FCR3 and HB3 parasites, even after 3 and up to 6 panning cycles respectively, did not show marked differences from the unselected stock (Figure 4.9).

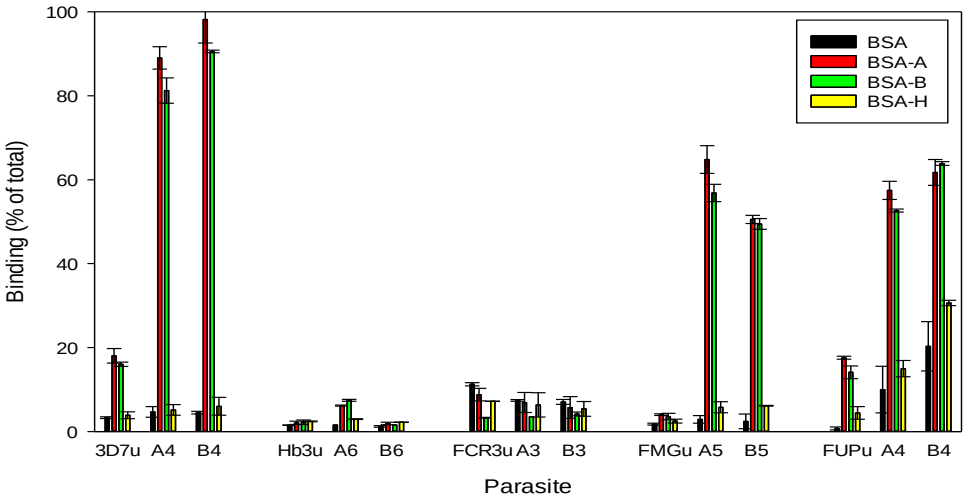


Figure 4.9 **Percentage adhesion of selected and unselected 3D7, HB3, FCR3, FMG and FUP parasites.** Following a minimum of 3 rounds of selection, blood group A and B selected parasites of 3D7 (A4, B4), FMG (A5, B5) and FUP (A4, B4 last 2 cluster of columns) show binding several fold higher than unselected parasites 3D7u, FMGu and FUPu respectively. Error bars represent standard deviation of triplicate readings.

Over time, the phenotype of selected parasites may diminish gradually. To investigate this phenomenon in the current study, the selected laboratory strains were monitored using this new assay method and re-selected by panning periodically to restore an optimum binding phenotype in the selected parasites. Older cultures maintained over 12 weeks without subsequent selection were tested in comparison to the more recently selected parasites.

A comparison of the binding capacity of selected parasites over a period of 4 months (16 weeks) without further reselection indicated that these selected parasites consistently bound to the A- and B-BSA constructs over the period, thus maintaining the acquired binding phenotype. This was in contrast to binding to BSA and the blood group O antigen construct H-BSA which was, as expected, recorded at low levels (less than 15%) over the same period (Figure 4.10).

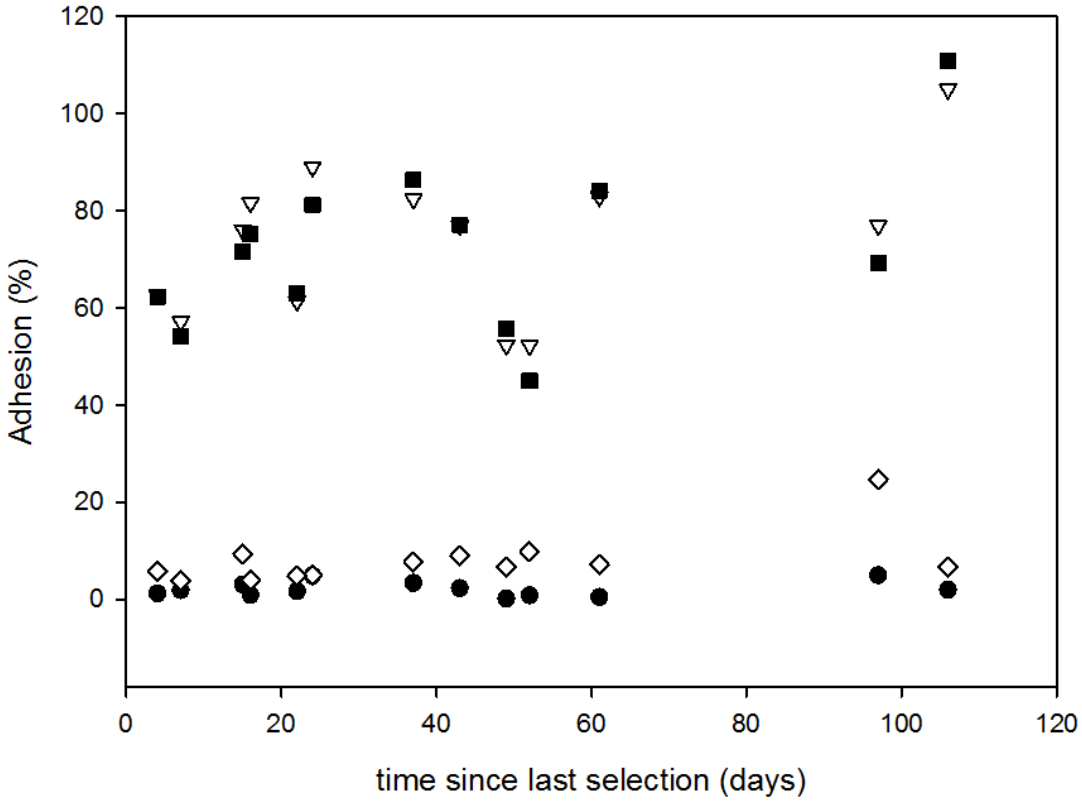


Figure 4.10 **Phenotype stability over a 16-week period**. Binding of A-BSA selected parasites of 3D7 to BSA and H-BSA (open diamond and black circles respectively) remain at low levels over the period, while binding to the A- and B-BSA constructs (open triangles and black squares respectively) are maintained at between 50-90%.

4.2.2 Binding of selected parasites to primary human ECs

Selected parasites (3D7, HB3 and FMG) were tested on ECs to investigate binding characteristics of IE containing these parasites on human aorta and dermal ECs.

All three parasite strains showed some binding to both aorta and dermal EC, with 3D7 and FMG selected parasites binding at a higher rate than their unselected isogenic lines. The HB3 strain however showed no difference between selected and unselected parasites in binding to the EC (Figure 4.11)

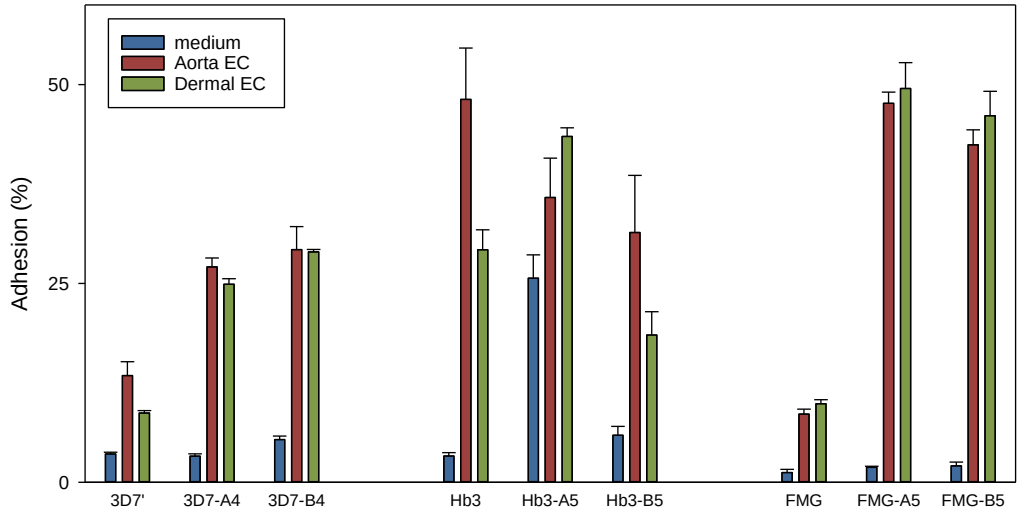


Figure 4.11 **Adhesion of A and B selected parasites to human Aorta and Dermal endothelial cells (ECs)**. Parasites selected on A and B carbohydrates adhered better (3D7-A4 & B4, FMG-A5 & B5) to the ECs than unselected lines, except for HB3, where selection (Hb3-A5, Hb3-B5) does not seem to affect binding characteristics. Error bars indicate standard deviation of duplicate measurements

4.2.3 Comparison of adhesion and rosetting in selected parasites

Blood group A and B selected 3D7, FMG and FUP parasites previously tested for binding to BSA-coupled blood group oligosaccharides were also tested for rosetting using RBC from donors of blood type O, A and AB. Blood groups A and B selected parasites bound significantly better than the corresponding unselected 3D7, FMG and FUP parasites, with unselected parasites binding at 20% or below (Figure 4.12). Results from the rosetting assays also showed rosetting only in selected parasites and in non-O RBCs (blood groups A and AB RBC). Rosetting was not observed in unselected 3D7, FMG or FUP parasites (Figure 4.12).

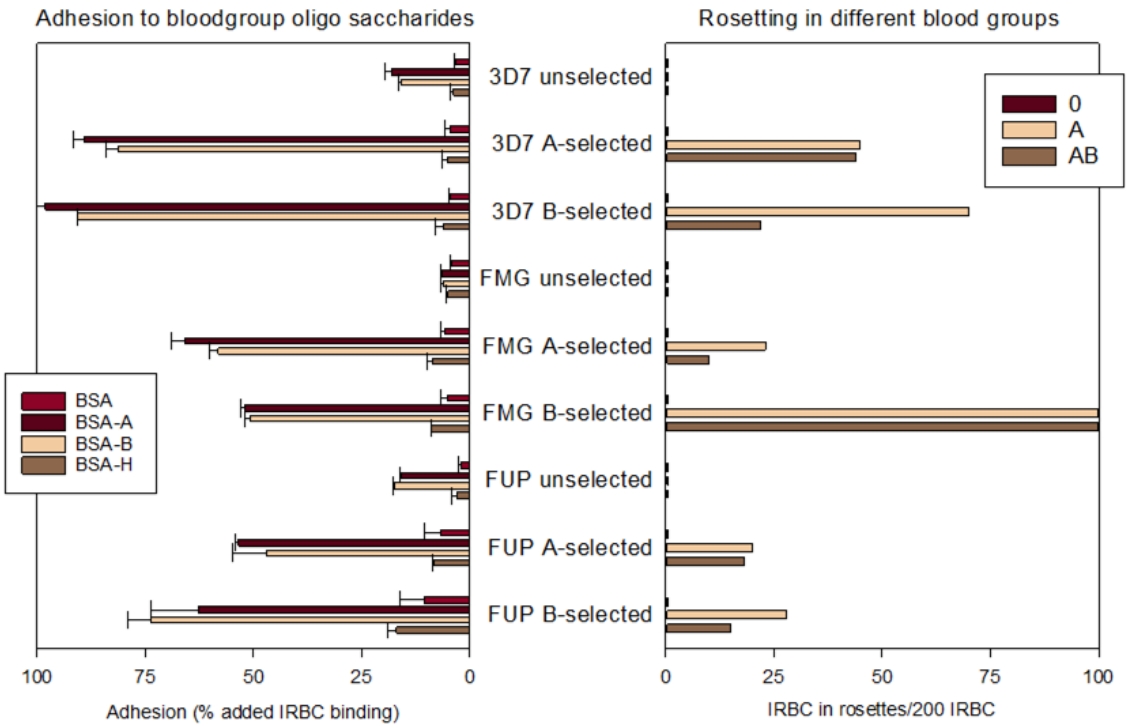


Figure 4.12 **Adhesion and rosetting characteristics of selected and unselected 3D7, FMG and FUP parasite strains**. Blood group A and B-selected parasites of all strains had much higher adhesion and formed rosettes, while the corresponding unselected parasites showed much lower adhesion and formed no rosettes in donor RBC from blood type A, AB and O. The error bars on the adhesion panel show the standard deviation of duplicate readings.

4.3 PfEMP1 and RIFIN expression patterns in selected *P. falciparum* laboratory parasite strains

The *var* and *rif* expression profiles of ring stage parasites selected on blood group antigens (Section 3.6) were investigated in 3D7 and FMG following storage in TRIzol and subsequent RNA extraction, cDNA generation and QPCR experiments and analysis.

4.3.1 QPCR *var* and *rif* transcripts in 3D7 selected and unselected parasites

To determine if any of the 58 *var* or 154 *rif* genes of 3D7 were expressed at a higher level in the blood group A and B adhesive parasites compared to the unselected control, QPCR experiments including primer sets designed to detect all the known var and rif sequences of 3D7 were performed. The experiments included cDNA prepared from both the A-BSA and B-BSA selected 3D7 following 3 rounds of panning, as well as from the unselected parental 3D7 that had been cultured in parallel. The results show that there was no single *var* gene that was clearly dominant before or after selection (Figure 4.13)

One *var* gene, namely PF13_0003, was transcribed at a slightly higher rate in both selected lines compared to the control, with fold increases of 1.3 and 1.7 respectively in transcription for the A-BSA and the B-BSA selected lines (Table 4.2). With the exception of PFE1640w/3D7var1 that showed a minor increase in transcription in the A-BSA selected 3D7 (but not in the B-BSA selected), transcription of all other *var* genes was decreased in the selected parasites. Therefore, the transcription of PF13_0003 was more prominent relative to the other *var* genes in the selected parasites. Consequently, in unselected 3D7 this transcript was the 10th most highly transcribed *var*

gene, constituting 3.5% of all *var* transcripts, whereas in the A and B selected lines it was the most dominant or second most dominant *var* gene constituting 18.4% and 18.2% of *var* transcripts respectively (Figure 4.13 and Table 4.2).

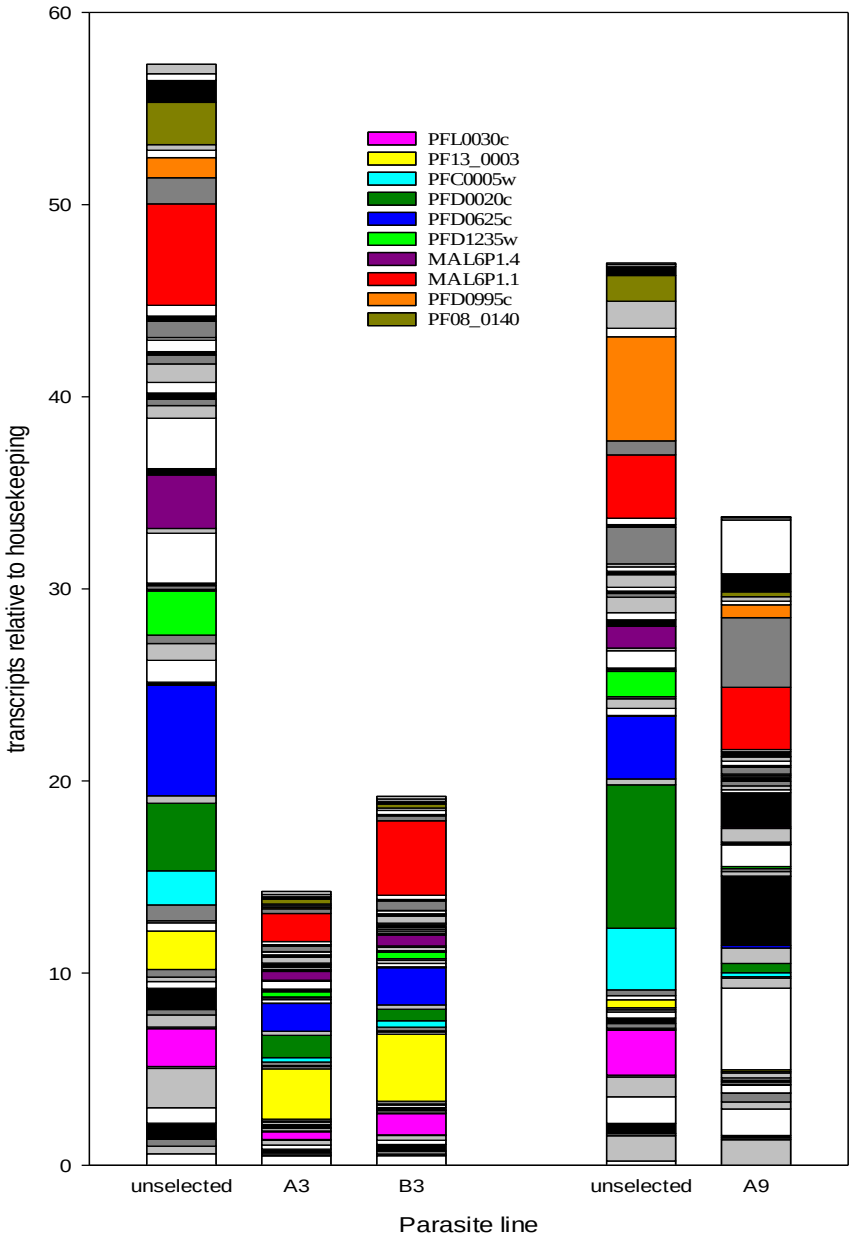


Figure 4.13 **Transcription levels of *var* genes in blood group antigen-selected and unselected 3D7 parasites.** Transcripts of *var* genes relative to housekeeping genes as measured by QPCR are shown, with the 10 highest average transcripts indicated in colour (also indicated by the same colour scheme in Table 4.2)

Table 4.2 Transcription levels of 58 *var* genes of 3D7, in 3D7 cultures

Table 4.2		3D7 (exp 1)			3D7 BGA3 (exp 1)				3D7 BGB3 (exp1)				3D7 (exp2)			3D7 BGA9 (exp2)			
Gene name	Type	Abs. #	relative	%	Abs. #	relative	%	Fold.	Abs. #	relative	%	fold	Abs. #	relative	%	Abs. #	relative	%	fold
PFE1640w	A/var1	2324	0.03	0.05	3466	0.04	0.29	1.55	854	0.01	0.07	0.54	7264	0.01	0.02	5100	0.01	0.02	0.82
PFL0030c	var2	172825	1.97	3.43	34066	0.40	2.82	0.20	66002	1.10	5.75	0.56	1709906	2.33	4.96	257683	0.41	1.21	0.18
PF11_0008	A/DC5	70931	0.81	1.41	18407	0.22	1.52	0.27	12840	0.21	1.12	0.27	1019305	1.39	2.95	233575	0.37	1.10	0.27
PF13_0003	A/DC5	176254	2.01	3.50	221654	2.61	18.35	1.30	208968	3.49	18.19	1.74	301536	0.41	0.87	2669053	4.24	12.57	10.34
MAL6P1.314	A	74746	0.85	1.48	24394	0.29	2.02	0.34	29689	0.50	2.58	0.58	1395762	1.90	4.05	153609	0.24	0.72	0.13
PFI1820w	A	33403	0.38	0.66	7030	0.08	0.58	0.22	12840	0.21	1.12	0.56	324057	0.44	0.94	150621	0.24	0.71	0.54
PFA0015c	A	25875	0.29	0.51	7654	0.09	0.63	0.31	7506	0.13	0.65	0.43	1032742	1.41	2.99	160814	0.26	0.76	0.18
PFD1235w	A	200917	2.29	3.99	22699	0.27	1.88	0.12	19912	0.33	1.73	0.15	967282	1.32	2.80	711033	1.13	3.35	0.86
PFD0020c	A	309538	3.52	6.15	98408	1.16	8.15	0.33	35664	0.60	3.11	0.17	5485119	7.47	15.90	505833	0.80	2.38	0.11
PF08_0140	DC8	193176	2.20	3.84	21682	0.26	1.79	0.12	12591	0.21	1.10	0.10	986472	1.34	2.86	588053	0.94	2.77	0.70
MAL6P1.316		119770	1.36	2.38	21260	0.25	1.76	0.18	15224	0.25	1.33	0.19	540065	0.74	1.57	423859	0.67	2.00	0.92
PF11_0521		179751	2.05	3.57	21682	0.26	1.79	0.13	14830	0.25	1.29	0.12	754197	1.03	2.19	282423	0.45	1.33	0.44
PF08_0141		227537	2.59	4.52	34066	0.40	2.82	0.16	9439	0.16	0.82	0.06	657299	0.89	1.91	443741	0.71	2.09	0.79
PFL0020w		8783	0.10	0.17	2371	0.03	0.20	0.28	2149	0.04	0.19	0.36	70931	0.10	0.21	11563	0.02	0.05	0.19
MAL6P1.4		244531	2.78	4.86	38077	0.45	3.15	0.16	34066	0.57	2.97	0.20	832038	1.13	2.41	1117169	1.78	5.26	1.57
PFA0005w		52828	0.60	1.05	42007	0.50	3.48	0.82	29689	0.50	2.58	0.83	164005	0.22	0.48	837505	1.33	3.95	5.97
PF10_0001		34515	0.39	0.69	3823	0.05	0.32	0.11	4808	0.08	0.42	0.20	967282	1.32	2.80	62633	0.10	0.30	0.08
PF10_0406		31907	0.36	0.63	9195	0.11	0.76	0.30	9689	0.16	0.84	0.45	81923	0.11	0.24	76229	0.12	0.36	1.09
PF11_0007		73292	0.83	1.46	16148	0.19	1.34	0.23	20710	0.35	1.80	0.42	389270	0.53	1.13	865380	1.38	4.08	2.60
PFL0935c		7858	0.09	0.16	3676	0.04	0.30	0.49	1973	0.03	0.17	0.37	32116	0.04	0.09	16795	0.03	0.08	0.61
PFL2665c		30278	0.34	0.60	10012	0.12	0.83	0.34	8119	0.14	0.71	0.39	230536	0.31	0.67	159765	0.25	0.75	0.81
PFL0005w		20983	0.24	0.42	4904	0.06	0.41	0.24	3225	0.05	0.28	0.23	84097	0.11	0.24	44267	0.07	0.21	0.61
PF13_0364		34515	0.39	0.69	8500	0.10	0.70	0.26	8389	0.14	0.73	0.36	79284	0.11	0.23	60616	0.10	0.29	0.89
PFB1055c		37337	0.42	0.74	9563	0.11	0.79	0.27	6938	0.12	0.60	0.27	157686	0.21	0.46	326186	0.52	1.54	2.42
PFB0010w		9255	0.11	0.18	4715	0.06	0.39	0.53	3310	0.06	0.29	0.53	3225	0.00	0.01	47885	0.08	0.23	17.35
PFC1120c		72814	0.83	1.45	16043	0.19	1.33	0.23	11413	0.19	0.99	0.23	217342	0.30	0.63	131270	0.21	0.62	0.71
PFC0005w		155634	1.77	3.09	19145	0.23	1.58	0.13	20175	0.34	1.76	0.19	2356802	3.21	6.83	305511	0.49	1.44	0.15
PFD0005w		99706	1.13	1.98	14830	0.17	1.23	0.15	10550	0.18	0.92	0.16	271542	0.37	0.79	145769	0.23	0.69	0.63
PFD1245c		5852	0.07	0.12	2294	0.03	0.19	0.41	1146	0.02	0.10	0.29	15628	0.02	0.05	13890	0.02	0.07	1.04
PFE0005w		16905	0.19	0.34	3849	0.05	0.32	0.24	1935	0.03	0.17	0.17	56035	0.08	0.16	40125	0.06	0.19	0.84
PF08_0142		22257	0.25	0.44	5410	0.06	0.45	0.25	4808	0.08	0.42	0.32	105759	0.14	0.31	52140	0.08	0.25	0.58
PF07_0139		84649	0.96	1.68	25875	0.31	2.14	0.32	21967	0.37	1.91	0.38	483170	0.66	1.40	223110	0.35	1.05	0.54
PFI1830c		52483	0.60	1.04	13620	0.16	1.13	0.27	9626	0.16	0.84	0.27	164005	0.22	0.48	141074	0.22	0.66	1.00
PF13_0001		23454	0.27	0.47	6047	0.07	0.50	0.27	4593	0.08	0.40	0.29	102352	0.14	0.30	69551	0.11	0.33	0.79
MAL6P1.1		464554	5.29	9.22	123757	1.46	10.24	0.28	232051	3.88	20.20	0.73	2419350	3.29	7.01	2280887	3.63	10.75	1.10
PFI0005w		31286	0.36	0.62	9882	0.12	0.82	0.33	7264	0.12	0.63	0.34	84649	0.12	0.25	74258	0.12	0.35	1.02
PFA0765c		42841	0.49	0.85	13268	0.16	1.10	0.32	8119	0.14	0.71	0.28	52140	0.07	0.15	28174	0.04	0.13	0.63
PFL1950w		55306	0.63	1.10	11488	0.14	0.95	0.22	7216	0.12	0.63	0.19	54945	0.07	0.16	83002	0.13	0.39	1.76
PFL1955w		97128	1.11	1.93	13095	0.15	1.08	0.14	7408	0.12	0.64	0.11	213114	0.29	0.62	71397	0.11	0.34	0.39
PFD0635c		14259	0.16	0.28	1675	0.02	0.14	0.12	2685	0.04	0.23	0.28	22257	0.03	0.06	18168	0.03	0.09	0.95
PF07_0050		230536	2.62	4.58	4358	0.05	0.36	0.02	6759	0.11	0.59	0.04	278748	0.38	0.81	127041	0.20	0.60	0.53

Table 4.2continued		3D7 (exp 1)			3D7BGA3 (exp 1)				3D7 BGB3 (exp1)				3D7 (exp2)			3D7 BGA9 (exp2)			
Gene name	Type	Abs. #	relative	%	Abs. #	relative	%	Fold.	Abs. #	relative	%	fold	Abs. #	relative	%	Abs. #	relative	%	fold
MAL7P1.55		28359	0.32	0.56	6414	0.08	0.53	0.23	9075	0.15	0.79	0.47	95864	0.13	0.28	51462	0.08	0.24	0.63
PFD1005c		40654	0.46	0.81	4872	0.06	0.40	0.12	3977	0.07	0.35	0.14	62224	0.08	0.18	57900	0.09	0.27	1.09
PF08_0106		15526	0.18	0.31	5445	0.06	0.45	0.36	3398	0.06	0.30	0.32	74258	0.10	0.22	140153	0.22	0.66	2.20
PF08_0103		48517	0.55	0.96	13799	0.16	1.14	0.29	13095	0.22	1.14	0.40	246138	0.34	0.71	2040598	3.24	9.61	9.69
MAL7P1.50		100033	1.14	1.99	10311	0.12	0.85	0.11	8093	0.14	0.70	0.12	331570	0.45	0.96	1761042	2.80	8.30	6.20
PFL1960w		24716	0.28	0.49	4416	0.05	0.37	0.19	2279	0.04	0.20	0.14	176254	0.24	0.51	64295	0.10	0.30	0.43
PFD0615c		33623	0.38	0.67	18049	0.21	1.49	0.56	13531	0.23	1.18	0.59	223110	0.30	0.65	83548	0.13	0.39	0.44
PFD0625c		505833	5.75	10.04	122949	1.45	10.18	0.25	115912	1.94	10.09	0.34	2403559	3.27	6.97	2251209	3.58	10.61	1.09
PFD1000c		77234	0.88	1.53	8172	0.10	0.68	0.11	9439	0.16	0.82	0.18	369403	0.50	1.07	100361	0.16	0.47	0.32
PFD1015c		39088	0.44	0.78	4003	0.05	0.33	0.11	5305	0.09	0.46	0.20	76730	0.10	0.22	66002	0.10	0.31	1.00
MAL6P1.252		11870	0.14	0.24	2309	0.03	0.19	0.20	1686	0.03	0.15	0.21	55669	0.08	0.16	30477	0.05	0.14	0.64
PF07_0048		29112	0.33	0.58	4623	0.05	0.38	0.16	5776	0.10	0.50	0.29	246138	0.34	0.71	101684	0.16	0.48	0.48
PF07_0049		57522	0.65	1.14	9377	0.11	0.78	0.17	5481	0.09	0.48	0.14	588053	0.80	1.70	147691	0.23	0.70	0.29
MAL7P1.56		29495	0.34	0.59	6207	0.07	0.51	0.22	6499	0.11	0.57	0.32	141074	0.19	0.41	131270	0.21	0.62	1.09
PF07_0051		48200	0.55	0.96	6127	0.07	0.51	0.13	3183	0.05	0.28	0.10	140153	0.19	0.41	53524	0.09	0.25	0.45
PF08_0107		12508	0.14	0.25	810	0.01	0.07	0.07	584	0.01	0.05	0.07	116674	0.16	0.34	14830	0.02	0.07	0.15
PFD0995c		91569	1.04	1.82	6007	0.07	0.50	0.07	4082	0.07	0.36	0.07	3979560	5.42	11.54	114404	0.18	0.54	0.03
Total *var*		5036654	57.30	100.00	1207986	14.25	100.00	0.25	1148536	19.20	100.00	0.34	34497669	46.95	100.00	21227210	33.75	100.00	0.72
House-keeping		87897			84788				59827				734699			628878			

Classification as UPS-A *var* genes and Domain Cassette (DC) group according to Rask *et al.* (2010). For all cultures tested the absolute number of detected transcripts (Abs #), the number of transcripts relative to the average transcript number of two house-keeping genes (relative) and the percentage of all *var* transcripts detected (%) are given. For the selected parasites, fold change in transcription relative to the unselected control parasite is given (fold). The 10 most highly transcribed genes are highlighted in colors corresponding to the color-coding of Figure 4.13.

In the first experiment, the total transcription of *rif* genes were approximately two-fold lower in the selected parasites than in the control (Figure 4.14). For the A-BSA selected parasite there was no obviously dominant *rif* transcript, whereas one gene, PF13_0004, was dominant in the B-BSA selected 3D7 (constituting 17.7% of all *rif* transcripts and 1.3 fold upregulated relative to the control parasite (Table 4.3)). In all three 3D7 parasite lines, approximately 2/3 of the *rif* transcripts were of group A. The PFA0020w group A RIFIN reported by Goel *et al.* (2015) to be expressed in blood group A-preferring rosetting 3D7, was transcribed at a very low level in all the 3 sublines of 3D7.

To confirm the results obtained in the first QPCR experiment, a second experiment including only unselected and A-BSA selected 3D7 was done. Seen in isolation, the positive fold change observed for PF13_0003 in the first experiment appeared to be much more prominent with a 10.3-fold higher transcription of the gene in A-BSA selected versus unselected 3D7 (Table 4.2). However, comparing the level of transcription with the first experiment, it was obvious that transcription had decreased in the unselected 3D7 but had been maintained at roughly the same level in the A-BSA selected line (Figure 4.13, yellow stacks, and Table 4.2). Consistent with the first experiment, no single dominant *var* transcript was detected. The overall decrease in transcription in the selected versus unselected line was much less apparent than in the first experiment. For the *rif* genes it now appeared that the PF13_0004 gene was now very dominant in the A-BSA selected parasite, responsible for more than 1/3 of all *rif* transcripts, whereas it was not prominent in the first experiment (Table 4.3).

Two genes, not very prominently transcribed in the first experiment, were now clearly dominant in the unselected 3D7: PF11_0009 and PFD0025w, representing 19.5% and

14.5% of the total *rif* transcription respectively. No decrease in total transcription of *rif* genes was observed in the second experiment.

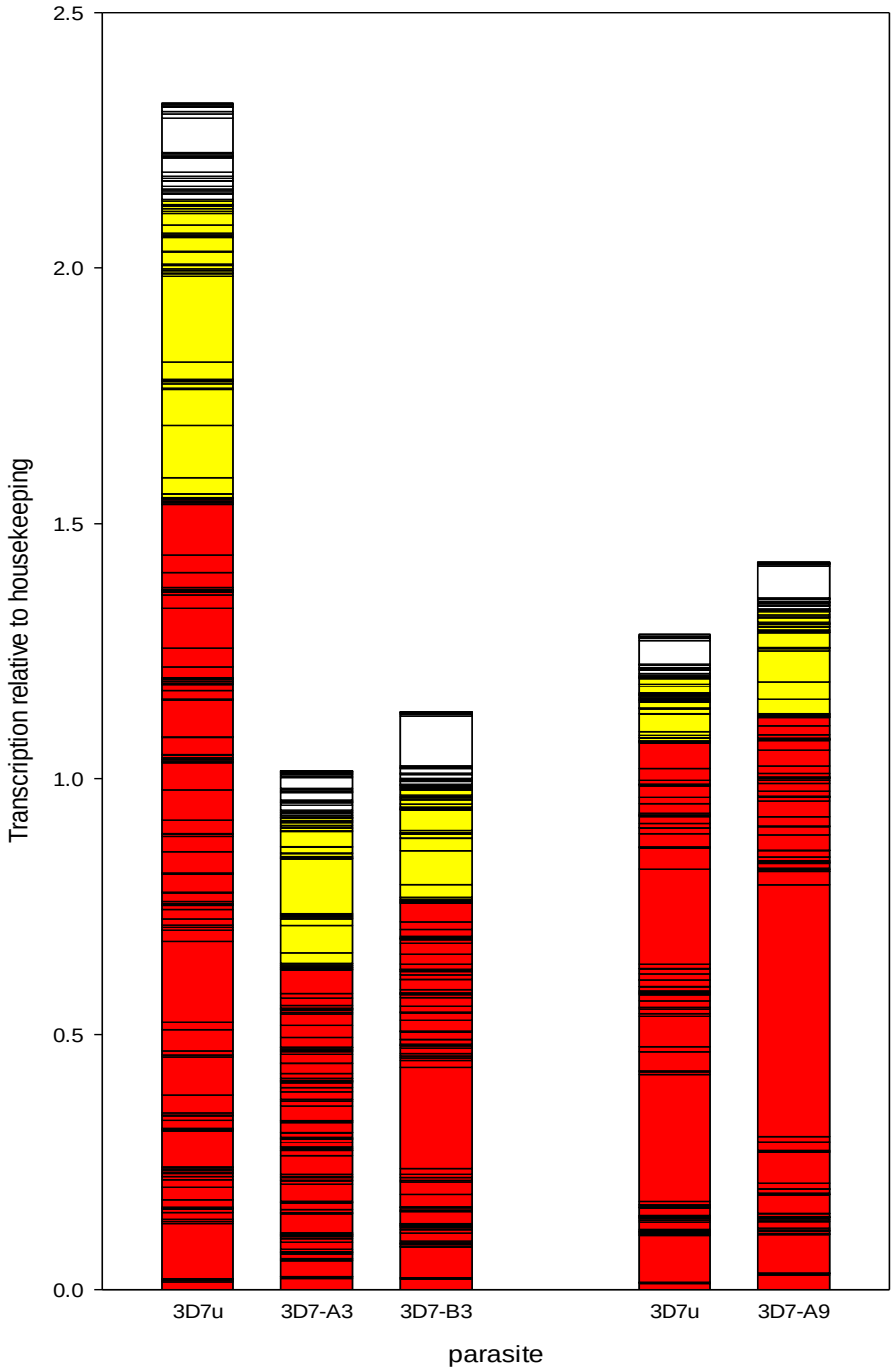


Figure 4.14 **The *rif* transcripts of selected and unselected parasites.** Relative to the housekeeping gene, total *rif* gene transcription was less in selected parasites (3D7-A3 and 3D7-B3) than in the unselected (3D7u)

Table 4.3 Transcription of the 154 *rif* genes of 3D7, in five 3D7 cultures

Table 4.3		3D7 (exp 1)			3D7 BGA3 (exp 1)				3D7 BGB3 (exp1)				3D7 (exp2)			3D7 BGA9 (exp2)			
Gene name	Type	Abs. #	relative	%	Abs. #	relative	%	Fold.	Abs. #	relative	%	fold	Abs. #	relative	%	Abs. #	relative	%	fold
MAL13P1.2	A	776	0.0153	0.66	1714	0.0219	2.16	1.44	1319	0.0205	1.81	1.34	6649	0.0121	0.94	16307	0.0287	2.02	2.39
MAL13P1.4	A	178	0.0035	0.15	206	0.0026	0.26	0.75	130	0.0020	0.18	0.58	687	0.0012	0.10	1313	0.0023	0.16	1.86
MAL13P1.500	A	122	0.0024	0.10	51	0.0007	0.06	0.27	43	0.0007	0.06	0.28	184	0.0003	0.03	363	0.0006	0.04	1.92
MAL13P1.515	A	9	0.0002	0.01	6	0.0001	0.01	0.45	4	0.0001	0.01	0.33	95	0.0002	0.01	252	0.0004	0.03	2.56
MAL13P1.520	A	2	0.0000	0.00	3	0.0000	0.00	0.77	1	0.0000	0.00	0.35	284	0.0005	0.04	193	0.0003	0.02	0.66
MAL13P1.535	A	5487	0.1079	4.64	2436	0.0312	3.07	0.29	3853	0.0598	5.29	0.55	50516	0.0916	7.13	42608	0.0751	5.27	0.82
MAL7P1.184	A	199	0.0039	0.17	130	0.0017	0.16	0.42	11	0.0002	0.02	0.04	704	0.0013	0.10	265	0.0005	0.03	0.37
MAL7P1.185	A	214	0.0042	0.18	189	0.0024	0.24	0.58	95	0.0015	0.13	0.35	1313	0.0024	0.19	836	0.0015	0.10	0.62
MAL7P1.213	A	678	0.0133	0.57	674	0.0086	0.85	0.65	235	0.0036	0.32	0.27	1357	0.0025	0.19	2399	0.0042	0.30	1.72
MAL7P1.216	A	343	0.0067	0.29	198	0.0025	0.25	0.38	75	0.0012	0.10	0.17	434	0.0008	0.06	783	0.0014	0.10	1.75
MAL7P1.217	A	62	0.0012	0.05	69	0.0009	0.09	0.72	36	0.0006	0.05	0.46	248	0.0004	0.03	367	0.0006	0.05	1.44
MAL7P1.222	A	113	0.0022	0.10	100	0.0013	0.13	0.58	95	0.0015	0.13	0.66	895	0.0016	0.13	487	0.0009	0.06	0.53
MAL7P1.57	A	740	0.0145	0.63	397	0.0051	0.50	0.35	209	0.0032	0.29	0.22	1551	0.0028	0.22	1901	0.0034	0.24	1.19
MAL8P1.208	A	1244	0.0244	1.05	1056	0.0135	1.33	0.55	1009	0.0157	1.39	0.64	7883	0.0143	1.11	7288	0.0128	0.90	0.90
MAL8P1.218	A	750	0.0147	0.63	540	0.0069	0.68	0.47	424	0.0066	0.58	0.45	2015	0.0037	0.28	1887	0.0033	0.23	0.91
MAL8P1.219	A	311	0.0061	0.26	336	0.0043	0.42	0.70	251	0.0039	0.34	0.64	1775	0.0032	0.25	2189	0.0039	0.27	1.20
PF07_0003	A	312	0.0061	0.26	78	0.0010	0.10	0.16	153	0.0024	0.21	0.39	976	0.0018	0.14	398	0.0007	0.05	0.40
PF07_0132	A	130	0.0025	0.11	113	0.0014	0.14	0.57	86	0.0013	0.12	0.53	413	0.0007	0.06	316	0.0006	0.04	0.74
PF07_0134	A	220	0.0043	0.19	109	0.0014	0.14	0.32	65	0.0010	0.09	0.23	688	0.0012	0.10	306	0.0005	0.04	0.43
PF07_0138	A	71	0.0014	0.06	63	0.0008	0.08	0.58	68	0.0011	0.09	0.76	405	0.0007	0.06	405	0.0007	0.05	0.97
PF08_0104	A	165	0.0032	0.14	145	0.0019	0.18	0.57	114	0.0018	0.16	0.55	434	0.0008	0.06	3372	0.0059	0.42	7.55
PF08_0138	A	75	0.0015	0.06	56	0.0007	0.07	0.49	28	0.0004	0.04	0.30	424	0.0008	0.06	294	0.0005	0.04	0.67
PF08_0139	A	3656	0.0719	3.09	2907	0.0372	3.67	0.52	1510	0.0235	2.07	0.33	8075	0.0146	1.14	20598	0.0363	2.55	2.48
PF10_0004	A	141	0.0028	0.12	107	0.0014	0.13	0.49	55	0.0009	0.08	0.31	406	0.0007	0.06	225	0.0004	0.03	0.54
PF10_0393	A	129	0.0025	0.11	54	0.0007	0.07	0.27	35	0.0005	0.05	0.21	288	0.0005	0.04	401	0.0007	0.05	1.36
PF10_0396	A	789	0.0155	0.67	472	0.0060	0.59	0.39	115	0.0018	0.16	0.12	1142	0.0021	0.16	1084	0.0019	0.13	0.92
PF10_0398	A	455	0.0089	0.38	1024	0.0131	1.29	1.47	297	0.0046	0.41	0.52	1384	0.0025	0.20	4499	0.0079	0.56	3.16
PF10_0400	A	60	0.0012	0.05	53	0.0007	0.07	0.58	22	0.0003	0.03	0.29	91	0.0002	0.01	163	0.0003	0.02	1.74
PF10_0403	A	227	0.0045	0.19	194	0.0025	0.24	0.56	88	0.0014	0.12	0.31	77	0.0001	0.01	314	0.0006	0.04	3.95
PF10_0405	A	1783	0.0350	1.51	2624	0.0336	3.31	0.96	1554	0.0241	2.13	0.69	3616	0.0066	0.51	6187	0.0109	0.77	1.66
PF11_0009	A	3786	0.0744	3.20	488	0.0062	0.61	0.08	1574	0.0244	2.16	0.33	137877	0.2499	19.46	34178	0.0603	4.23	0.24
PF11_0010	A	184	0.0036	0.16	107	0.0014	0.13	0.38	195	0.0030	0.27	0.84	2901	0.0053	0.41	763	0.0013	0.09	0.26
PF11_0021	A	411	0.0081	0.35	398	0.0051	0.50	0.63	358	0.0056	0.49	0.69	1042	0.0019	0.15	1311	0.0023	0.16	1.22
PF11_0520	A	2109	0.0415	1.78	189	0.0024	0.24	0.06	405	0.0063	0.56	0.15	20598	0.0373	2.91	10289	0.0181	1.27	0.49
PF11_0529	A	743	0.0146	0.63	319	0.0041	0.40	0.28	724	0.0112	0.99	0.77	5369	0.0097	0.76	6080	0.0107	0.75	1.10
PF13_0004	A	8022	0.1577	6.79	2851	0.0365	3.59	0.23	12854	0.1996	17.66	1.27	32789	0.0594	4.63	279053	0.4919	34.51	8.28
PF13_0005	A	1105	0.0217	0.94	792	0.0101	1.00	0.47	856	0.0133	1.18	0.61	2838	0.0051	0.40	14879	0.0262	1.84	5.10
PF14_0004	A	13	0.0003	0.01	8	0.0001	0.01	0.39	4	0.0001	0.01	0.22	35	0.0001	0.01	16	0.0000	0.00	0.43
PF14_0006	A	278	0.0055	0.24	193	0.0025	0.24	0.45	315	0.0049	0.43	0.89	4931	0.0089	0.70	1834	0.0032	0.23	0.36
PF14_0769	A	224	0.0044	0.19	163	0.0021	0.21	0.48	255	0.0040	0.35	0.90	1818	0.0033	0.26	472	0.0008	0.06	0.25
PF14_0772	A	624	0.0123	0.53	160	0.0020	0.20	0.17	296	0.0046	0.41	0.37	7084	0.0128	1.00	1170	0.0021	0.14	0.16

Table 4.3continued		3D7 (exp 1)			3D7 BGA3 (exp 1)				3D7 BGB3 (exp1)				3D7 (exp2)			3D7 BGA9 (exp2)			
Gene name	Type	Abs. #	relative	%	Abs. #	relative	%	Fold.	Abs. #	relative	%	fold	Abs. #	relative	%	Abs. #	relative	%	fold
PFA0010c	A	934	0.0184	0.79	768	0.0098	0.97	0.54	687	0.0107	0.94	0.58	6449	0.0117	0.91	5264	0.0093	0.65	0.79
PFA0020w	A	398	0.0078	0.34	559	0.0072	0.71	0.92	302	0.0047	0.42	0.60	1372	0.0025	0.19	945	0.0017	0.12	0.67
PFA0040w	A	83	0.0016	0.07	51	0.0006	0.06	0.40	38	0.0006	0.05	0.36	825	0.0015	0.12	229	0.0004	0.03	0.27
PFA0045c	A	94	0.0018	0.08	57	0.0007	0.07	0.39	57	0.0009	0.08	0.48	831	0.0015	0.12	748	0.0013	0.09	0.88
PFA0050c	A	220	0.0043	0.19	176	0.0023	0.22	0.52	100	0.0016	0.14	0.36	1198	0.0022	0.17	1065	0.0019	0.13	0.86
PFA0080c	A	847	0.0166	0.72	719	0.0092	0.91	0.55	583	0.0091	0.80	0.54	4618	0.0084	0.65	3895	0.0069	0.48	0.82
PFA0740w	A	69	0.0014	0.06	14	0.0002	0.02	0.14	14	0.0002	0.02	0.16	138	0.0002	0.02	336	0.0006	0.04	2.37
PFA0760w	A	1799	0.0354	1.52	1527	0.0195	1.93	0.55	934	0.0145	1.28	0.41	6796	0.0123	0.96	7022	0.0124	0.87	1.00
PFB0030c	A	22	0.0004	0.02	97	0.0012	0.12	2.90	54	0.0008	0.07	1.94	73	0.0001	0.01	256	0.0005	0.03	3.42
PFB0060w	A	75	0.0015	0.06	165	0.0021	0.21	1.43	46	0.0007	0.06	0.48	111	0.0002	0.02	206	0.0004	0.03	1.81
PFB1010w	A	18	0.0004	0.02	27	0.0003	0.03	0.97	12	0.0002	0.02	0.53	79	0.0001	0.01	51	0.0001	0.01	0.64
PFB1050w	A	2109	0.0415	1.78	2237	0.0286	2.82	0.69	1360	0.0211	1.87	0.51	6506	0.0118	0.92	16924	0.0298	2.09	2.53
PFC0010c	A	1524	0.0299	1.29	722	0.0092	0.91	0.31	926	0.0144	1.27	0.48	5463	0.0099	0.77	8986	0.0158	1.11	1.60
PFC0040w	A	206	0.0041	0.17	264	0.0034	0.33	0.84	79	0.0012	0.11	0.30	254	0.0005	0.04	550	0.0010	0.07	2.11
PFC1095w	A	74	0.0015	0.06	45	0.0006	0.06	0.40	30	0.0005	0.04	0.32	120	0.0002	0.02	262	0.0005	0.03	2.12
PFC1115w	A	1338	0.0263	1.13	1129	0.0144	1.42	0.55	728	0.0113	1.00	0.43	4643	0.0084	0.66	10255	0.0181	1.27	2.15
PFD0025w	A	3001	0.0590	2.54	653	0.0084	0.82	0.14	1088	0.0169	1.49	0.29	102352	0.1855	14.45	17468	0.0308	2.16	0.17
PFD0030c	A	2708	0.0532	2.29	731	0.0094	0.92	0.18	368	0.0057	0.50	0.11	22898	0.0415	3.23	3985	0.0070	0.49	0.17
PFD0040c	A	89	0.0017	0.07	152	0.0019	0.19	1.11	54	0.0008	0.07	0.48	418	0.0008	0.06	249	0.0004	0.03	0.58
PFD0045c	A	205	0.0040	0.17	107	0.0014	0.14	0.34	51	0.0008	0.07	0.20	306	0.0006	0.04	306	0.0005	0.04	0.97
PFD0050w	A	38	0.0007	0.03	19	0.0002	0.02	0.33	6	0.0001	0.01	0.13	10	0.0000	0.00	6	0.0000	0.00	0.56
PFD0055w	A	155	0.0030	0.13	61	0.0008	0.08	0.26	27	0.0004	0.04	0.14	101	0.0002	0.01	109	0.0002	0.01	1.05
PFD0060w	A	283	0.0056	0.24	329	0.0042	0.41	0.76	80	0.0012	0.11	0.22	363	0.0007	0.05	520	0.0009	0.06	1.39
PFD0640c	A	34	0.0007	0.03	19	0.0002	0.02	0.35	10	0.0002	0.01	0.24	32	0.0001	0.00	55	0.0001	0.01	1.70
PFD0645w	A	1701	0.0334	1.44	731	0.0094	0.92	0.28	403	0.0063	0.55	0.19	14012	0.0254	1.98	5602	0.0099	0.69	0.39
PFD1230c	A	52	0.0010	0.04	3	0.0000	0.00	0.04	6	0.0001	0.01	0.10	127	0.0002	0.02	141	0.0002	0.02	1.08
PFD1240w	A	3660	0.0719	3.10	1542	0.0197	1.94	0.27	1275	0.0198	1.75	0.28	6180	0.0112	0.87	8463	0.0149	1.05	1.33
PFE0020c	A	104	0.0020	0.09	37	0.0005	0.05	0.23	19	0.0003	0.03	0.14	150	0.0003	0.02	91	0.0002	0.01	0.59
PFF0015c	A	870	0.0171	0.74	1359	0.0174	1.71	1.02	558	0.0087	0.77	0.51	4623	0.0084	0.65	3289	0.0058	0.41	0.69
PFF0025w	A	679	0.0133	0.57	399	0.0051	0.50	0.38	424	0.0066	0.58	0.49	7506	0.0136	1.06	2053	0.0036	0.25	0.27
PFF0035c	A	160	0.0031	0.14	164	0.0021	0.21	0.67	31	0.0005	0.04	0.15	550	0.0010	0.08	266	0.0005	0.03	0.47
PFF0855c	A	180	0.0035	0.15	124	0.0016	0.16	0.45	61	0.0009	0.08	0.27	747	0.0014	0.11	733	0.0013	0.09	0.95
PFF1555w	A	183	0.0036	0.15	295	0.0038	0.37	1.05	145	0.0022	0.20	0.62	277	0.0005	0.04	384	0.0007	0.05	1.35
PFF1560c	A	102	0.0020	0.09	70	0.0009	0.09	0.45	26	0.0004	0.04	0.20	109	0.0002	0.02	149	0.0003	0.02	1.32
PFF1565c	A	66	0.0013	0.06	43	0.0005	0.05	0.42	21	0.0003	0.03	0.25	52	0.0001	0.01	178	0.0003	0.02	3.33
PFF1575w	A	1066	0.0210	0.90	1470	0.0188	1.85	0.90	644	0.0100	0.88	0.48	1922	0.0035	0.27	3977	0.0070	0.49	2.01
PFF1590w	A	1881	0.0370	1.59	1844	0.0236	2.33	0.64	1262	0.0196	1.73	0.53	10188	0.0185	1.44	8208	0.0145	1.02	0.78
PFI0010c	A	3951	0.0777	3.34	1731	0.0222	2.18	0.29	1377	0.0214	1.89	0.28	6803	0.0123	0.96	17583	0.0310	2.17	2.51
PFI0020w	A	1318	0.0259	1.11	246	0.0032	0.31	0.12	430	0.0067	0.59	0.26	12292	0.0223	1.74	10573	0.0186	1.31	0.84
PFI0030c	A	271	0.0053	0.23	366	0.0047	0.46	0.88	95	0.0015	0.13	0.28	1030	0.0019	0.15	1016	0.0018	0.13	0.96
PFI0035c	A	47	0.0009	0.04	42	0.0005	0.05	0.59	19	0.0003	0.03	0.32	94	0.0002	0.01	87	0.0002	0.01	0.90
PFI0050c	A	76	0.0015	0.06	95	0.0012	0.12	0.82	36	0.0006	0.05	0.37	158	0.0003	0.02	346	0.0006	0.04	2.13
PFI0065w	A	80	0.0016	0.07	69	0.0009	0.09	0.56	53	0.0008	0.07	0.52	144	0.0003	0.02	268	0.0005	0.03	1.81
PFI0070w	A	58	0.0011	0.05	72	0.0009	0.09	0.81	71	0.0011	0.10	0.97	119	0.0002	0.02	217	0.0004	0.03	1.78
PFI0075w	A	218	0.0043	0.18	406	0.0052	0.51	1.21	155	0.0024	0.21	0.56	439	0.0008	0.06	751	0.0013	0.09	1.66

Table 4.3continued		3D7 (exp 1)			3D7 BGA3 (exp 1)				3D7 BGB3 (exp1)				3D7 (exp2)			3D7 BGA9 (exp2)			
Gene name	Type	Abs. #	relative	%	Abs. #	relative	%	Fold.	Abs. #	relative	%	fold	Abs. #	relative	%	Abs. #	relative	%	fold
PFI1815c	A	1483	0.0291	1.25	1134	0.0145	1.43	0.50	861	0.0134	1.18	0.46	4172	0.0076	0.59	3782	0.0067	0.47	0.88
PFL0010c	A	1735	0.0341	1.47	680	0.0087	0.86	0.26	956	0.0148	1.31	0.44	12618	0.0229	1.78	9968	0.0176	1.23	0.77
PFL0025c	A	5034	0.0989	4.26	3605	0.0461	4.54	0.47	2340	0.0363	3.21	0.37	27626	0.0501	3.90	9195	0.0162	1.14	0.32
PFL2615w	A	51	0.0010	0.04	76	0.0010	0.10	0.98	41	0.0006	0.06	0.64	141	0.0003	0.02	274	0.0005	0.03	1.88
PFL2625w	A	159	0.0031	0.13	75	0.0010	0.09	0.31	105	0.0016	0.14	0.52	954	0.0017	0.13	256	0.0005	0.03	0.26
PFL2640c	A	133	0.0026	0.11	189	0.0024	0.24	0.93	97	0.0015	0.13	0.58	184	0.0003	0.03	435	0.0008	0.05	2.29
PFL2645c	A	189	0.0037	0.16	83	0.0011	0.11	0.29	120	0.0019	0.17	0.50	575	0.0010	0.08	803	0.0014	0.10	1.36
PFL2660w	A	79	0.0016	0.07	233	0.0030	0.29	1.92	95	0.0015	0.13	0.95	320	0.0006	0.05	960	0.0017	0.12	2.92
PFB0040c	A	55	0.0011	0.05	74	0.0010	0.09	0.87	56	0.0009	0.08	0.80	74	0.0001	0.01	335	0.0006	0.04	4.39
PF10_0003	B1	358	0.0070	0.30	241	0.0031	0.30	0.44	223	0.0035	0.31	0.49	3186	0.0058	0.45	980	0.0017	0.12	0.30
PF10_0397	B1	43	0.0009	0.04	51	0.0007	0.06	0.77	16	0.0003	0.02	0.30	87	0.0002	0.01	146	0.0003	0.02	1.64
PF10_0404	B1	1592	0.0313	1.35	1602	0.0205	2.02	0.66	1602	0.0249	2.20	0.80	2635	0.0048	0.37	16060	0.0283	1.99	5.93
PF13_0006	B1	5184	0.1019	4.39	4150	0.0531	5.23	0.52	4260	0.0661	5.85	0.65	3938	0.0071	0.56	20374	0.0359	2.52	5.03
PF14_0003	B1	3585	0.0705	3.03	1026	0.0131	1.29	0.19	1551	0.0241	2.13	0.34	19041	0.0345	2.69	34327	0.0605	4.25	1.75
PF14_0770	B1	122	0.0024	0.10	103	0.0013	0.13	0.55	55	0.0009	0.08	0.36	326	0.0006	0.05	122	0.0002	0.02	0.36
PFA0095c	B1	429	0.0084	0.36	144	0.0018	0.18	0.22	482	0.0075	0.66	0.89	5128	0.0093	0.72	2384	0.0042	0.29	0.45
PFA0745w	B1	225	0.0044	0.19	150	0.0019	0.19	0.43	67	0.0010	0.09	0.24	708	0.0013	0.10	786	0.0014	0.10	1.08
PFB1015w	B1	86	0.0017	0.07	107	0.0014	0.13	0.81	48	0.0007	0.07	0.44	255	0.0005	0.04	190	0.0003	0.02	0.72
PFB1040w	B1	140	0.0027	0.12	147	0.0019	0.19	0.69	59	0.0009	0.08	0.33	124	0.0002	0.02	271	0.0005	0.03	2.12
PFD1010w	B1	1715	0.0337	1.45	138	0.0018	0.17	0.05	276	0.0043	0.38	0.13	36	0.0001	0.01	18	0.0000	0.00	0.47
PFI0025c	B1	8518	0.1674	7.21	8353	0.1069	10.53	0.64	2570	0.0399	3.53	0.24	5981	0.0108	0.84	16504	0.0291	2.04	2.68
PFL0015c	B1	258	0.0051	0.22	72	0.0009	0.09	0.18	79	0.0012	0.11	0.24	1636	0.0030	0.23	216	0.0004	0.03	0.13
PFL2605w	B1	58	0.0011	0.05	56	0.0007	0.07	0.63	16	0.0003	0.02	0.22	49	0.0001	0.01	55	0.0001	0.01	1.08
PFL2655w	B1	14	0.0003	0.01	12	0.0001	0.01	0.54	3	0.0001	0.00	0.19							
MAL13P1.530	B2	237	0.0047	0.20	73	0.0009	0.09	0.20	55	0.0008	0.08	0.18	955	0.0017	0.13	298	0.0005	0.04	0.30
MAL7P1.215	B2	178	0.0035	0.15	141	0.0018	0.18	0.51	55	0.0009	0.08	0.25	409	0.0007	0.06	406	0.0007	0.05	0.97
PF11_0020	B2	340	0.0067	0.29	463	0.0059	0.58	0.89	128	0.0020	0.18	0.30	945	0.0017	0.13	1196	0.0021	0.15	1.23
PF14_0766	B2	132	0.0026	0.11	84	0.0011	0.11	0.41	48	0.0007	0.07	0.28	920	0.0017	0.13	807	0.0014	0.10	0.85
PFA0030c	B2	1204	0.0237	1.02	908	0.0116	1.15	0.49	351	0.0055	0.48	0.23	2156	0.0039	0.30	3387	0.0060	0.42	1.53
PFC0030c	B2	75	0.0015	0.06	64	0.0008	0.08	0.55	22	0.0003	0.03	0.23	224	0.0004	0.03	226	0.0004	0.03	0.98
PFF1570w	B2	1371	0.0269	1.16	2314	0.0296	2.92	1.10	537	0.0083	0.74	0.31	1148	0.0021	0.16	2471	0.0044	0.31	2.09
PFI0015c	B2	111	0.0022	0.09	100	0.0013	0.13	0.59	103	0.0016	0.14	0.74	271	0.0005	0.04	457	0.0008	0.06	1.64
PFI1810w	B2	153	0.0030	0.13	388	0.0050	0.49	1.66	190	0.0029	0.26	0.98	410	0.0007	0.06	496	0.0009	0.06	1.18
MAL7P1.219	B3	157	0.0031	0.13	179	0.0023	0.23	0.74	88	0.0014	0.12	0.44	371	0.0007	0.05	498	0.0009	0.06	1.31
PF10_0394	B3	908	0.0179	0.77	258	0.0033	0.33	0.19	211	0.0033	0.29	0.18	1084	0.0020	0.15	976	0.0017	0.12	0.88
PF11_0515	B3	1147	0.0225	0.97	418	0.0054	0.53	0.24	641	0.0099	0.88	0.44	7563	0.0137	1.07	4877	0.0086	0.60	0.63
PF14_0005	B3	211	0.0041	0.18	158	0.0020	0.20	0.49	132	0.0021	0.18	0.50	2490	0.0045	0.35	1063	0.0019	0.13	0.42
PF14_0008	B3	271	0.0053	0.23	164	0.0021	0.21	0.39	177	0.0027	0.24	0.52	6040	0.0109	0.85	2218	0.0039	0.27	0.36
PFB0015c	B3	271	0.0053	0.23	371	0.0048	0.47	0.89	181	0.0028	0.25	0.53	434	0.0008	0.06	3770	0.0066	0.47	8.44
PFB0055c	B3	20	0.0004	0.02	30	0.0004	0.04	1.00	14	0.0002	0.02	0.55	24	0.0000	0.00	86	0.0002	0.01	3.46
PFB1005w	B3	89	0.0018	0.08	61	0.0008	0.08	0.45	14	0.0002	0.02	0.12	90	0.0002	0.01	245	0.0004	0.03	2.65
PFC1100w	B3	15	0.0003	0.01	24	0.0003	0.03	1.00	13	0.0002	0.02	0.66	16	0.0000	0.00	35	0.0001	0.00	2.08
PFE1630w	B3	352	0.0069	0.30	254	0.0032	0.32	0.47	170	0.0026	0.23	0.38	521	0.0009	0.07	1099	0.0019	0.14	2.05
PF10_0402	B3	170	0.0033	0.14	106	0.0014	0.13	0.41	32	0.0005	0.04	0.15	131	0.0002	0.02	210	0.0004	0.03	1.56

Table 4.3continued		3D7 (exp 1)			3D7 BGA3 (exp 1)				3D7 BGB3 (exp1)				3D7 (exp2)			3D7 BGA9 (exp2)			
Gene name	Type	Abs. #	relative	%	Abs. #	relative	%	Fold.	Abs. #	relative	%	fold	Abs. #	relative	%	Abs. #	relative	%	fold
MAL13P1.495		518	0.0102	0.44	330	0.0042	0.42	0.41	345	0.0054	0.47	0.53	627	0.0011	0.09	1191	0.0021	0.15	1.85
MAL7P1.200		127	0.0025	0.11	63	0.0008	0.08	0.32	19	0.0003	0.03	0.12	431	0.0008	0.06	154	0.0003	0.02	0.35
PF08_0105		202	0.0040	0.17	220	0.0028	0.28	0.71	93	0.0014	0.13	0.36	591	0.0011	0.08	3138	0.0055	0.39	5.17
PF10_0002		188	0.0037	0.16	139	0.0018	0.18	0.48	133	0.0021	0.18	0.56	898	0.0016	0.13	309	0.0005	0.04	0.33
PF10_0005		267	0.0052	0.23	746	0.0096	0.94	1.82	153	0.0024	0.21	0.45	1954	0.0035	0.28	908	0.0016	0.11	0.45
PF10_0006		510	0.0100	0.43	388	0.0050	0.49	0.49	469	0.0073	0.64	0.73	3737	0.0068	0.53	2128	0.0038	0.26	0.55
PF10_0399		42	0.0008	0.04	35	0.0004	0.04	0.53	7	0.0001	0.01	0.14	44	0.0001	0.01	130	0.0002	0.02	2.90
PF10_0401		242	0.0048	0.20	134	0.0017	0.17	0.36	62	0.0010	0.09	0.20	419	0.0008	0.06	308	0.0005	0.04	0.71
PF11_0517		207	0.0041	0.17	91	0.0012	0.11	0.29	44	0.0007	0.06	0.17	879	0.0016	0.12	393	0.0007	0.05	0.43
PF11_0519		408	0.0080	0.34	153	0.0020	0.19	0.24	66	0.0010	0.09	0.13	1274	0.0023	0.18	658	0.0012	0.08	0.50
PF14_0002		1415	0.0278	1.20	1140	0.0146	1.44	0.52	588	0.0091	0.81	0.33	2618	0.0047	0.37	2420	0.0043	0.30	0.90
PF14_0768		117	0.0023	0.10	58	0.0007	0.07	0.32	28	0.0004	0.04	0.19	196	0.0004	0.03	284	0.0005	0.04	1.41
PFA0710c		67	0.0013	0.06	112	0.0014	0.14	1.09	28	0.0004	0.04	0.33	141	0.0003	0.02	345	0.0006	0.04	2.39
PFB0035c		23	0.0004	0.02	79	0.0010	0.10	2.26	42	0.0007	0.06	1.46	30	0.0001	0.00	202	0.0004	0.03	6.58
PFB1035w		195	0.0038	0.16	275	0.0035	0.35	0.92	158	0.0024	0.22	0.64	328	0.0006	0.05	421	0.0007	0.05	1.25
PFC0035w		121	0.0024	0.10	118	0.0015	0.15	0.64	66	0.0010	0.09	0.44	402	0.0007	0.06	382	0.0007	0.05	0.92
PFD0070c		3421	0.0672	2.89	1643	0.0210	2.07	0.31	6248	0.0970	8.58	1.44	25371	0.0460	3.58	35200	0.0621	4.35	1.35
PFD1020c		422	0.0083	0.36	204	0.0026	0.26	0.31	218	0.0034	0.30	0.41	2314	0.0042	0.33	1805	0.0032	0.22	0.76
PFE0025c		234	0.0046	0.20	260	0.0033	0.33	0.72	93	0.0014	0.13	0.31	937	0.0017	0.13	597	0.0011	0.07	0.62
PFF1545w		442	0.0087	0.37	204	0.0026	0.26	0.30	91	0.0014	0.13	0.16	381	0.0007	0.05	278	0.0005	0.03	0.71
PFI0055c		71	0.0014	0.06	57	0.0007	0.07	0.52	15	0.0002	0.02	0.16	118	0.0002	0.02	231	0.0004	0.03	1.91
PFI1805w		163	0.0032	0.14	190	0.0024	0.24	0.76	54	0.0008	0.07	0.26	919	0.0017	0.13	537	0.0009	0.07	0.57
PFL2585c		65	0.0013	0.06	38	0.0005	0.05	0.38	38	0.0006	0.05	0.46	215	0.0004	0.03	155	0.0003	0.02	0.70
PFL2630w		103	0.0020	0.09	76	0.0010	0.10	0.48	36	0.0006	0.05	0.28	1848	0.0034	0.26	986	0.0017	0.12	0.52
Total *Rif*		118215	23236	100.00	79323	10153	100.00	0.44	64401	11304	100.00	0.49	708443	12842	100.00	808531	14253	100.00	1.11
House-keeping		50876			78124				72797				551682			567251			

Classification of A or B RIFINs according to Joannin *et al.* (2008). For all cultures tested the absolute number of detected transcripts (Abs #), the number of transcripts relative to the average transcript number of two house-keeping genes (relative) and the percentage of all *rif*-transcripts detected (%) are given. For the selected parasites, fold change in transcription relative to the unselected control parasite is given (fold). The 10 most highly transcribed genes are highlighted in color.

4.3.2 QPCR *var* transcripts in FMG selected and unselected parasites

From the results obtained from the 3D7 QPCR, PF13_0003 appeared to be the most likely candidate among the *var* genes as being the mediator of blood group A and B adhesion in this parasite. It would be expected that the blood group A and B binding FMG parasites express one or more *var* genes with similar structural motifs as those proposed to mediate binding between PF13_0003 and blood group A and B. To test this, a similar QPCR analysis was performed for the FMG parasites using primers generated to detect 56 *var* genes of the IT4 genotype to which the FMG strain belongs.

In contrast to what was observed for PF13_0003, the two SD3-loop containing UPS-A *var* genes of IT4 previously proposed to mediate blood group A dependent rosetting, IT4var9 and IT4var60, were transcribed at a very low level (<0.2% of all *var* transcripts in all FMG sublines), and were down-regulated in the selected relative to the unselected parasites (Table 4.4). The one DC5 type *var* gene of the IT4 genome, IT4var2, was transcribed at less than 1% in all sublines.

IT4var21, which has also been previously associated with rosetting and binding to blood group A, was transcribed at a slightly higher level but was down regulated in the selected parasites (Figure 4.15). As in the first 3D7 experiment, only one *var* gene was upregulated in both selected sublines, namely IT4var32b (Figure 4.15), with a 6.7- and 1.3- fold increased transcription in A and B-BSA selected parasites, respectively.

One *var* gene (IT4var19), which like IT4var32b belongs to the DC8 group of *var* genes, *was erroneously omitted from the analysis*, so a prominent expression of this gene cannot be ruled out in the selected parasites. The overall trend however, as for the 3D7 experiment, was a general decrease in *var* expression in the A and B-BSA selected parasites.

Table 4.4 Transcription of 56 *var* genes from the IT4/FCR3/FMG genome

Table 4.4		FMG			FMG BGA5				FMG BGB5			
Gene name	Type	Abs. #	relative	%*	Abs. #	relative	%*	Fold.	Abs. #	relative	%*	fold
IT4var35	A/var1	16272	0.16	0.57	17718	0.16	3.55	0.98	11081	0.14	3.02	0.88
IT4var04	var2	3585	0.04	0.13	3077	0.03	0.62	0.77	2495	0.03	0.68	0.89
IT4var02	A/DC5	2838	0.03	0.10	3712	0.03	0.74	1.18	2184	0.03	0.60	0.99
IT4var09	A/Rosetting/SD3 loop	1652	0.02	0.06	374	0.00	0.07	0.20	232	0.00	0.06	0.18
IT4var60	A/Rosetting/SD3 loop	3624	0.04	0.13	335	0.00	0.07	0.08	159	0.00	0.04	0.06
IT4var03	A	818	0.01	0.03	665	0.01	0.13	0.73	292	0.00	0.08	0.46
IT4var07	A	79892	0.81	2.82	1285	0.01	0.26	0.01	628	0.01	0.17	0.01
IT4var08	A	3350	0.03	0.12	1313	0.01	0.26	0.35	862	0.01	0.24	0.33
IT4var18	A	17411	0.18	0.61	1791	0.02	0.36	0.09	1322	0.02	0.36	0.10
IT4var22	A	807	0.01	0.03	345	0.00	0.07	0.39	181	0.00	0.05	0.29
IT4var64	A	2468	0.02	0.09	900	0.01	0.18	0.33	480	0.01	0.13	0.25
IT4var06	DC8	3329	0.03	0.12	3562	0.03	0.71	0.97	1912	0.02	0.52	0.74
IT4var20	DC8	6067	0.06	0.21	1345	0.01	0.27	0.20	735	0.01	0.20	0.16
IT4var32b	DC8	22184	0.22	0.78	164543	1.50	32.95	6.69	21517	0.28	5.87	1.25
IT4var21	Rosetting	35161	0.36	1.24	12522	0.11	2.51	0.32	9045	0.12	2.47	0.33
IT4var01		13905	0.14	0.49	2175	0.02	0.44	0.14	1544	0.02	0.42	0.14
IT4var05		9026	0.09	0.32	2410	0.02	0.48	0.24	1405	0.02	0.38	0.20
IT4var10		8697	0.09	0.31	3554	0.03	0.71	0.37	1733	0.02	0.47	0.26
IT4var11		12305	0.12	0.43	4232	0.04	0.85	0.31	2653	0.03	0.72	0.28
IT4var12		21283	0.22	0.75	3307	0.03	0.66	0.14	2506	0.03	0.68	0.15
IT4var13		5846	0.06	0.21	1337	0.01	0.27	0.21	648	0.01	0.18	0.14
IT4var14		65499	0.66	2.31	39302	0.36	7.87	0.54	5264	0.07	1.44	0.10
IT4var15		23429	0.24	0.83	5017	0.05	1.00	0.19	2578	0.03	0.70	0.14
IT4var16		18589	0.19	0.66	2933	0.03	0.59	0.14	1446	0.02	0.39	0.10
IT4var17		7730	0.08	0.27	2845	0.03	0.57	0.33	1430	0.02	0.39	0.24
IT4var23		13966	0.14	0.49	3432	0.03	0.69	0.22	2795	0.04	0.76	0.26
IT4var24		6992	0.07	0.25	1891	0.02	0.38	0.24	1217	0.02	0.33	0.22
IT4var25		10493	0.11	0.37	3179	0.03	0.64	0.27	2312	0.03	0.63	0.28
IT4var26		12332	0.12	0.44	3972	0.04	0.80	0.29	3350	0.04	0.91	0.35
IT4var27		1685817	17.07	59.47	37623	0.34	7.53	0.02	174151	2.27	47.54	0.13
IT4var28		20643	0.21	0.73	4793	0.04	0.96	0.21	3432	0.04	0.94	0.21
IT4var29		11203	0.11	0.40	4123	0.04	0.83	0.33	2332	0.03	0.64	0.27
IT4var30		47625	0.48	1.68	7068	0.06	1.42	0.13	4450	0.06	1.21	0.12
IT4var31		49210	0.50	1.74	9511	0.09	1.90	0.17	4963	0.06	1.35	0.13
IT4var32a		229783	2.33	8.11	9892	0.09	1.98	0.04	8967	0.12	2.45	0.05
IT4var33		10795	0.11	0.38	2506	0.02	0.50	0.21	1494	0.02	0.41	0.18
IT4var34		21098	0.21	0.74	10795	0.10	2.16	0.46	4866	0.06	1.33	0.30
IT4var36		26941	0.27	0.95	6976	0.06	1.40	0.23	5050	0.07	1.38	0.24
IT4var39		8325	0.08	0.29	2723	0.02	0.55	0.30	1729	0.02	0.47	0.27
IT4var40		7099	0.07	0.25	2358	0.02	0.47	0.30	1363	0.02	0.37	0.25
IT4var41		30711	0.31	1.08	4909	0.04	0.98	0.14	2175	0.03	0.59	0.09
IT4var44		26765	0.27	0.94	27960	0.26	5.60	0.94	18069	0.24	4.93	0.87
IT4var45		17335	0.18	0.61	4689	0.04	0.94	0.24	2567	0.03	0.70	0.19
IT4var46		15611	0.16	0.55	4996	0.05	1.00	0.29	2926	0.04	0.80	0.24
IT4var47		10379	0.11	0.37	3972	0.04	0.80	0.35	2119	0.03	0.58	0.26
IT4var51		8947	0.09	0.32	2901	0.03	0.58	0.29	1814	0.02	0.50	0.26
IT4var54		12092	0.12	0.43	1082	0.01	0.22	0.08	568	0.01	0.16	0.06
IT4var58		42701	0.43	1.51	13575	0.12	2.72	0.29	9165	0.12	2.50	0.28
IT4var59		29399	0.30	1.04	9785	0.09	1.96	0.30	7514	0.10	2.05	0.33
IT4var61		9408	0.10	0.33	3454	0.03	0.69	0.33	2595	0.03	0.71	0.35
IT4var62		24208	0.25	0.85	7193	0.07	1.44	0.27	3887	0.05	1.06	0.21
IT4var63		5584	0.06	0.20	2838	0.03	0.57	0.46	1446	0.02	0.39	0.33
IT4var65		25847	0.26	0.91	12413	0.11	2.49	0.43	7935	0.10	2.17	0.39
IT4var66		13997	0.14	0.49	3609	0.03	0.72	0.23	3037	0.04	0.83	0.28
IT4var67		11614	0.12	0.41	3660	0.03	0.73	0.28	2955	0.04	0.81	0.33
IT4var68		1993	0.02	0.07	869	0.01	0.17	0.39	764	0.01	0.21	0.49
Total *var**		2834680	28.70	100.00	499346	4.56	100.00	0.16	366342	4.77	100.00	0.17
House-keeping		98785			109517				76856			

Var gene transcripts in unselected, A- and B- selected FMG cultures. For all cultures tested the absolute number of detected transcripts (Abs #), the number of transcripts relative to the average transcript number of two house-keeping genes (relative) and the percentage of all *var* transcripts detected (%) are given. For the selected parasites, fold change in transcription relative to the unselected control parasite is given (fold). The 10 most highly transcribed genes are highlighted in colors corresponding to the color-coding of Figure 4.15. *The DC8 *var* gene IT4var19 was by error not included in the analysis.

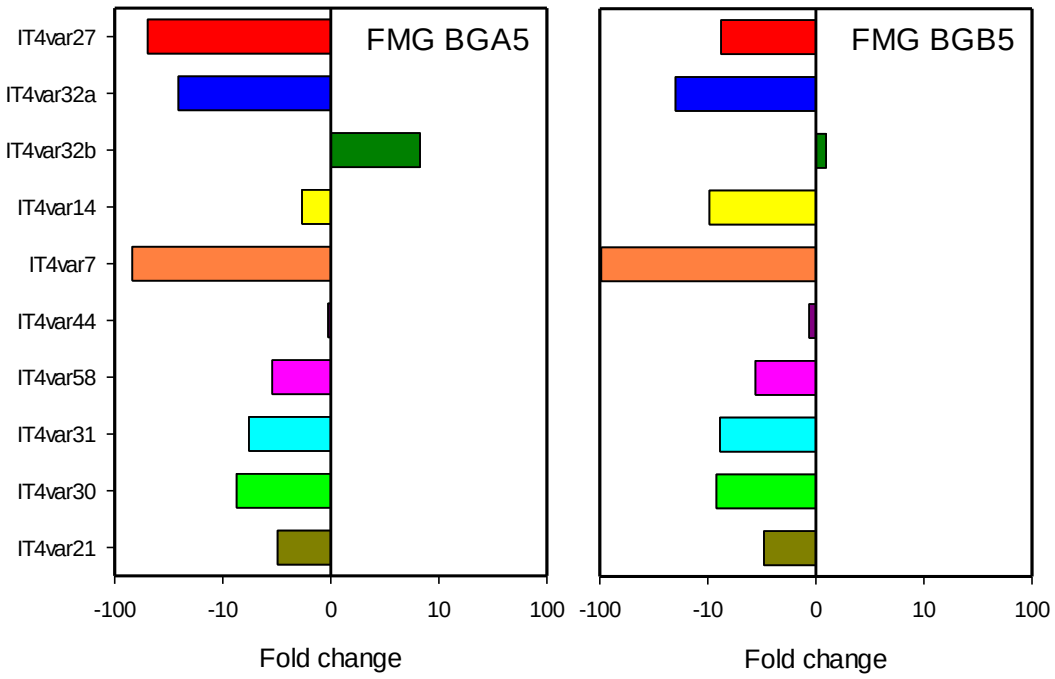


Figure 4.15 **Fold change of the top 10 *var* transcripts in the FMG A- and B-BSA selected parasites**. As shown, IT4var32b (dark green bar) was the only transcript upregulated in both selected parasites.

4.4 Fieldwork: Background and Demographics

A total of 113 patients, aged 5 months to 12 years, were recruited in 2016 after the inclusion criteria were met for this aspect of the study. There were 60 male and 53 females, representing 53% and 47% of the cohort respectively. The categories of disease included 53 (46.9%) cases of severe malaria (SM) and 52 (46.02%) cases of uncomplicated malaria (UM). Six (6) patients, or 5.31% of the cohort, were not categorized. Table 4.5 summarizes characteristics of recruited patients.

Table 4.5 Characteristics of patients recruited for the study

Number recruited	113
Age range	5 months to 12yrs
Gender:	
Male	60
Female	53
Parasite positive (Microscopy)	90 of 113
Parasitaemia Range (per µl)	0 to 433,525 parasites
Clinical category:	
Severe Malaria	53
Uncomplicated Malaria	52
Not categorized & febrile cases	8
**Blood group:*	
A	24
B	27
AB	4
O	57
*Sickling positive	6 of 112

*no blood group and sickling record for 1 patient

Tests to determine the blood type were done for each patient, and all blood types were observed among the patients recruited for the study. Blood group AB was the least common, while blood group O as expected, was the most common blood type recorded in the study (Table 4.5). The sickling positive patients were excluded from the analysis.

4.5 Plate-based ABO haem-release binding assays in clinical isolates

Following studies of the rosetting and binding characteristics of parasite lines selected on blood group carbohydrates, further investigations into the binding characteristics of clinical parasite isolates from patients were conducted. These field isolates, stored in liquid nitrogen during field work, were transported to the laboratory where they were subsequently thawed, cultured and used in *in vitro* experiments to study their binding profiles to immobilized blood group oligosaccharides on polystyrene plates.

In all, 39 field isolates were tested for their ability to bind the BSA-conjugated blood group oligosaccharides immobilized on 96 well Polysorb plates. Each plate included at least the unselected 3D7 laboratory strain and the blood group A ‘control’ parasites.

4.5.1 Relative adhesion of selected 3D7 parasites during experiments

The laboratory selected strains used on each plate as a reference during the assays showed consistent binding over the course of the experiments. The relative adhesion to blood group A polysaccharides for 3D7, 3D7-A and 3D7-B selected parasites for all assays combined show consistently higher rates of adhesion during the experiments. Relative adhesion of both 3D7-A and 3D7-B were comparable and significantly higher than for the unselected 3D7 parasites (Figure 4.16).

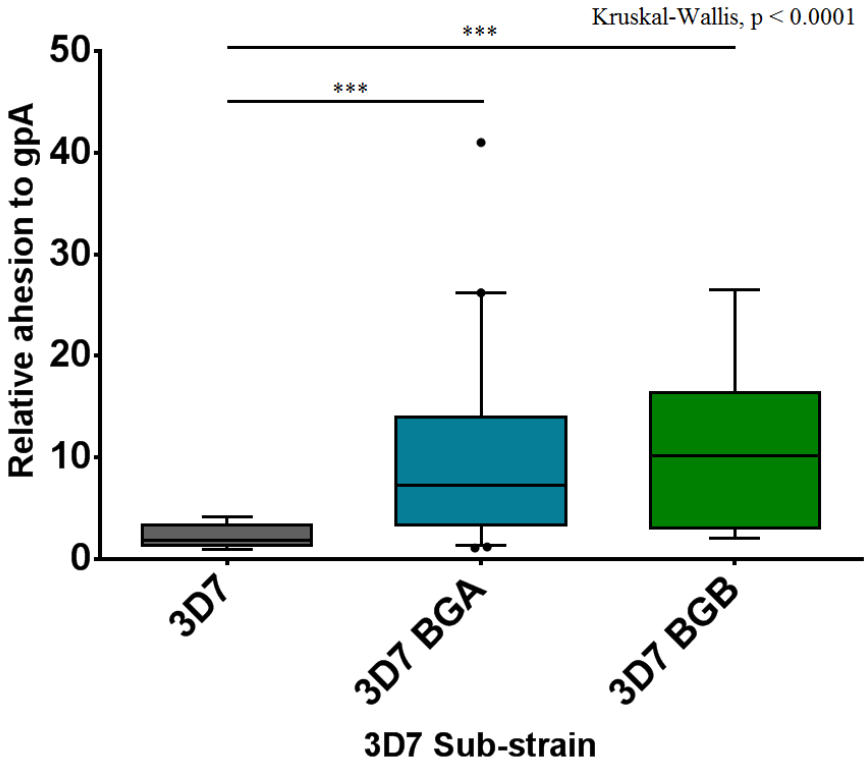


Figure 4.16 **Relative adhesion of 3D7 parasites to blood group A antigen in plate based assays**. Combined relative adhesion of 3D7 parasites over the course of the experiments indicate a significantly higher relative adhesion to A-BSA by the 3D7 BGA (blue box; n = 20) and 3D7 BGB (green box; n = 14) selected parasites ($p < 0.0001$, Kruskal-Wallis test and Dunn's post hoc test) over the unselected isogenic 3D7 line (grey box; n = 18).

4.5.2 Relative adhesion of field isolates and blood type of donor

Comparing relative adhesion of field isolates from patients based on grouping into blood type O and non-O indicates that parasite binding to blood group A is independent of the blood type of the patient donor. Thus, the binding of patient isolates to the blood group A construct, A-BSA, shows no significant difference (P = 0.4619, Mann Whitney test) between blood type (O versus non-O) and capability to bind blood group A antigen (Figure 4.17).

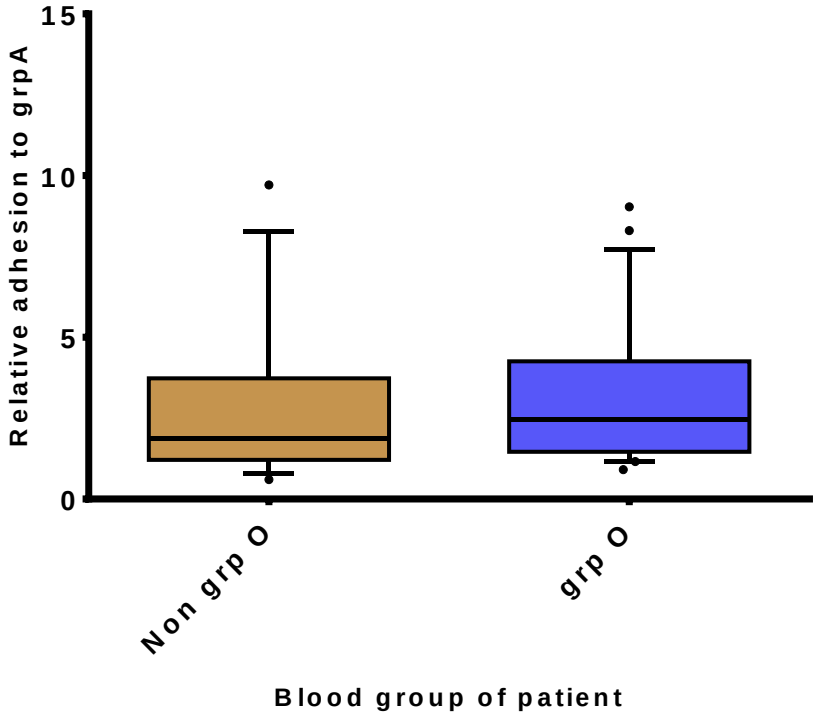


Figure 4.17 **Relative adhesion of non-group O and group O patient parasite isolates to blood group A antigen**. Boxplots of relative adhesion (median values) to blood group A antigen by field isolates from patients with blood group A and AB (Non grp O, brown box; n = 13) and from patients with blood group O (grp O, blue box; n = 26). Upper and lower limits of boxes are the 25th and 75th percentile respectively, while whiskers show the minimum and maximum values. Outliers are shown as data points beyond these values.

4.5.3 Summary of binding characteristics of field isolates

For most isolates, the binding to the A and B constructs were similar, and binding of clinical isolates to the blood group H construct was only observed in one isolate.

Of the 39 field isolates tested, 8 bound to at least one blood group oligosaccharide. Interestingly, a lone isolate bound to the blood group H antigen, though this was only slightly above the cut-off value. This isolate also happened to bind both the A and B construct above cut-off values. All 8 of the binding field isolates were observed in children 5 years and below. Figure 4.18 summarizes the binding characteristics of field isolates to the A- and B-BSA constructs. Five of the 8 field isolates that bound to ABO polysaccharides were from donors with blood type O, two were of blood type B, and the remaining binding isolate was a donor of blood type A.

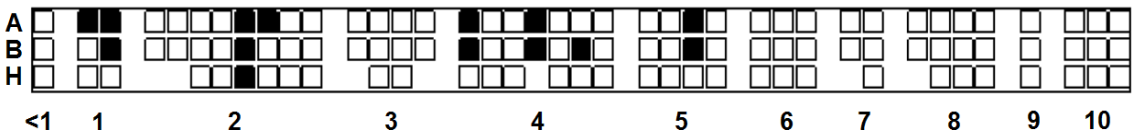


Figure 4.18 **Binding of field isolates to the blood group A, B and O (H) constructs in 39 children 10 years and below**. Black squares denote positive binding to blood group antigens (mean + 2SD of unselected 3D7), and was observed only in children 5 years and below. One isolate from a 2-year old patient bound to all three constructs.

4.6 Rosetting in field isolates

During field work, malaria parasites from vein-puncture blood samples of recruited patients were processed and cultured in the donor's RBC and monitored until they were at late trophozoite stages and then used for rosetting assays.

A total of 70 field isolates from the cohort were successfully assayed. At least a rosette was observed in 50 of the parasite cultures, representing 71% of the total assayed cohort.

4.6.1 Rosetting and blood groups

Table 4.6 shows the characteristics and distribution of the various blood groups in the sampled cohort. Using the Kruskal Wallis test, the differences observed in rosetting rates between the different blood groups were found to be statistically insignificant ($p = 0.3533$), though the mean rosetting rate in blood group O was about half of those observed for both blood groups A and B.

Table 4.6 Characteristics of field isolates in the different blood groups

Blood group	Total isolates	Rosetting isolates	Range (rosettes/200 IE)	Mean # of rosettes	Median	KW test p value
A	13	9	0-105	19.9	7	P > 0.05
B	18	15	0-127	21	12	
AB	2	2	4-13	8.5	8.5	
O	37	24	0-86	10.9	7	

The total number of rosetting isolates, as well as rosetting rates for each patient blood type in the field isolates are shown for the various blood groups. The Kruskal-Wallis (KW) test for the difference in medians for these groups showed no significant difference between the blood types.

A comparison of mean rosetting rates between patients of blood group O and non-group O categories showed no significant difference ($p>0.05$) (Figure 4.19). In other tests, results of a comparison between parasite density (number of parasites per microlitre) in rosetting and non-rosetting isolates showed there was no significant difference in parasite load (as detected by microscopy) between these groups (Mann-Whitney, $p = 0.112$).

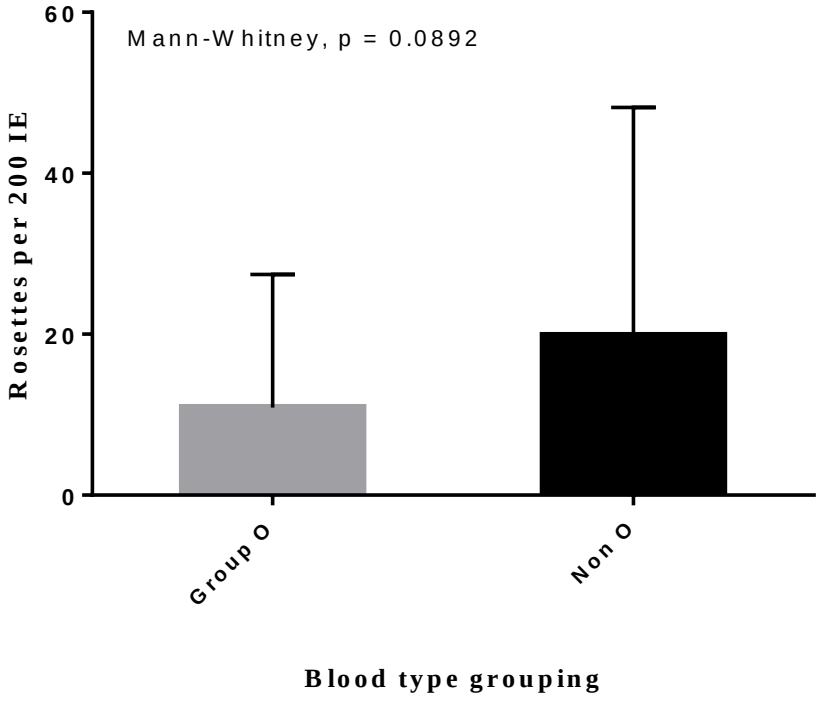
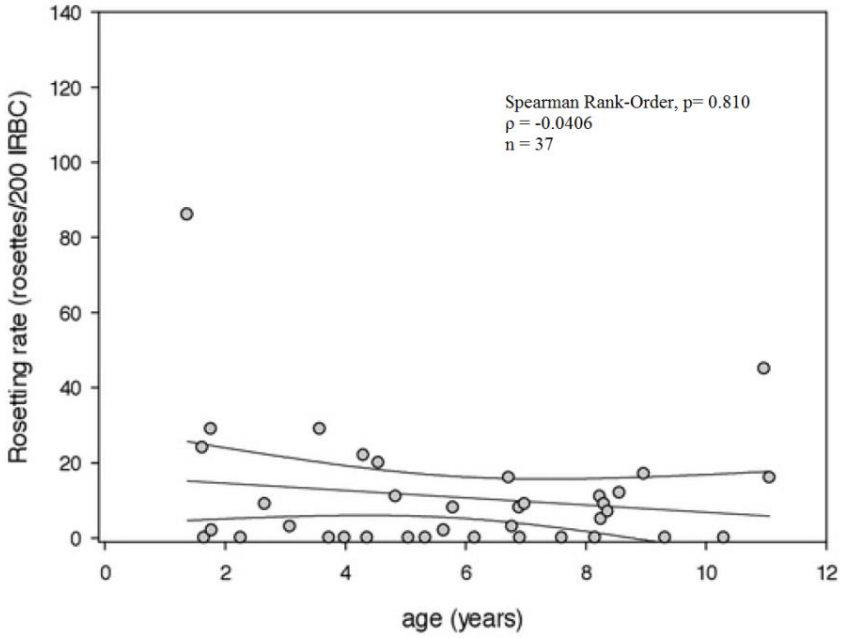


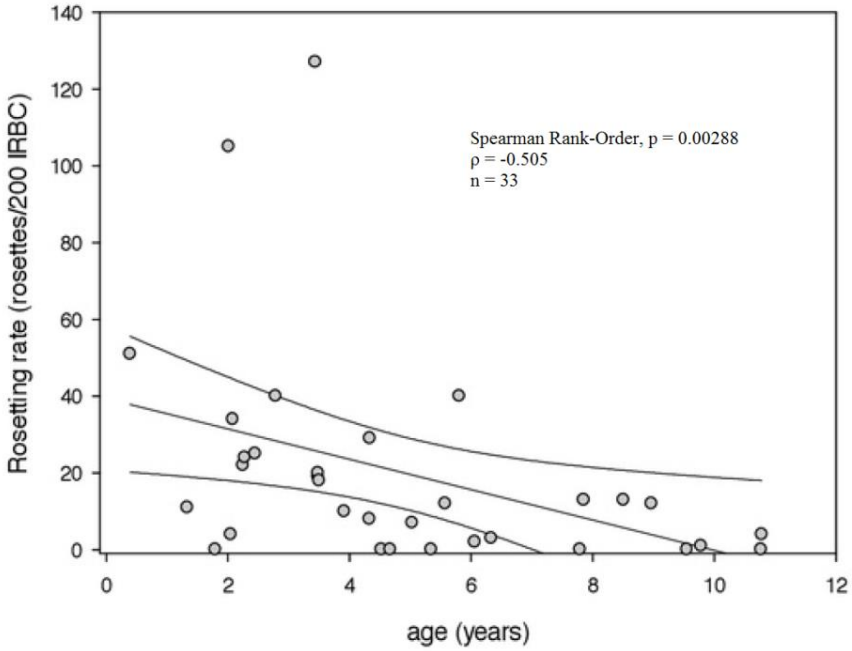
Figure 4.19 **Rosetting rates in blood group O versus non-group O malaria patients**. Mean values of the number of rosettes counted per 200 IE in blood group O (grey bar, n = 37) and non-O (black bar, n = 33) patients were statistically insignificant ($p > 0.05$). Error bars are SD of mean number of rosettes

4.6.2 Rosetting and Age

In general, rosetting rates were found to decline with age (n=70, ρ= -0.322, p=0.00657, Spearman Rank order). This observed effect was due to non-O blood types, since no significant change in rosetting rates were observed for blood type O when patients were grouped according to blood type (Figure 4.20, A). On the other hand, in patients with blood types other than group O, the rosetting rates declined significantly with increasing age (Figure 4.20, B).



A. Rosetting rates and age in blood type O



B. Rosetting rate and age in non-O blood types

Figure 4.20 **Correlation between rosetting and age in blood groups.** Panel A shows the correlation between rosetting rates and age in patients of blood group O. In Panel B, this correlation is more evident and significant in patients with non-O blood types.

4.6.3 Rosetting and Parasitaemia

To determine whether the level of parasitaemia observed in patients had any effect on, or association with, the rosetting rate, tests of association were conducted on these parameters. The tests showed no significant association between parasitaemia and rosetting for the cohort, even for the higher risk age group of children 5 years and below, as well as in patients over 5 years of age (Figure 4.21, A and B respectively). There is a gradual increase in rosetting rate as the parasite density increases in the 0-5 year group, though this association was not significant (P = 0.235). This association was much less obvious in the 5years+ group (P= 0.83: Figure 4.21, B)

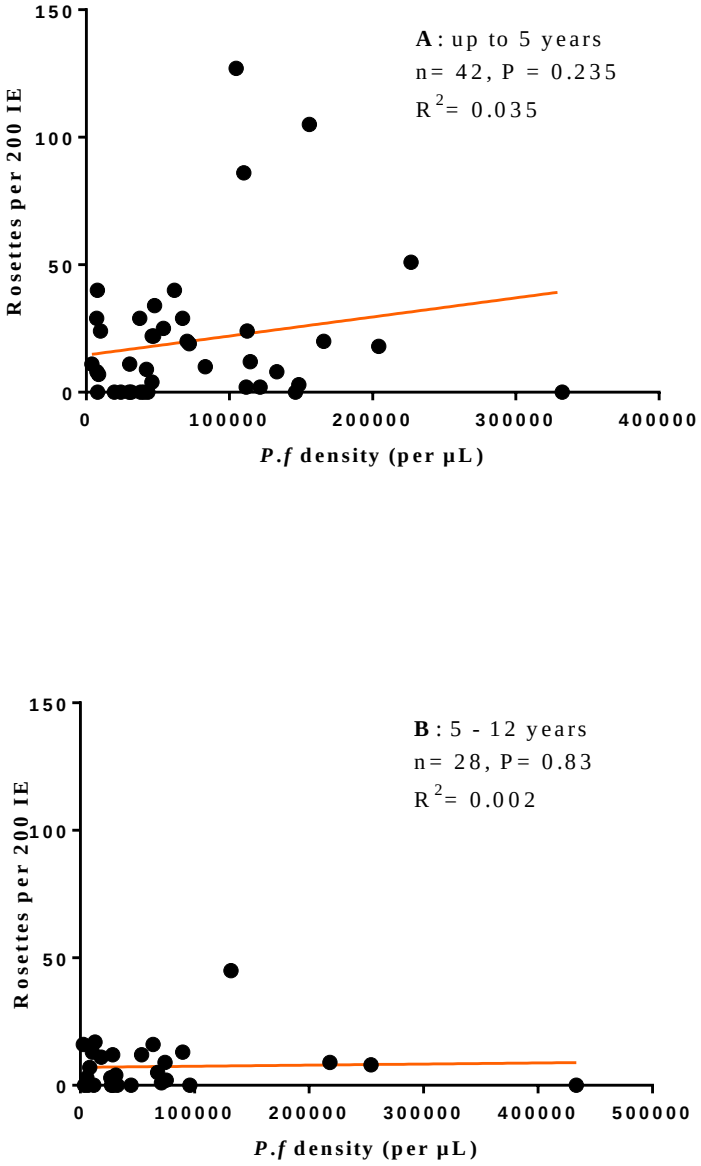


Figure 4.21 **Association between parasitaemia and rosetting isolates in 0-5, and 5 -12 year groups**. Rosetting rates versus parasite density showing the line of best fit for the 0 - 5 year group (Graph A), and the 5 - 12 year group (Graph B) Each point represents an individual patient record.

However, when analyzed solely on the basis of the age category of 0-5 and 5 plus years, the rosetting rates in field isolates from children 5 years and below were significantly higher ($p<0.05$) than rosetting rates recorded in isolates from children over 5 years of age (Figure 4.22).

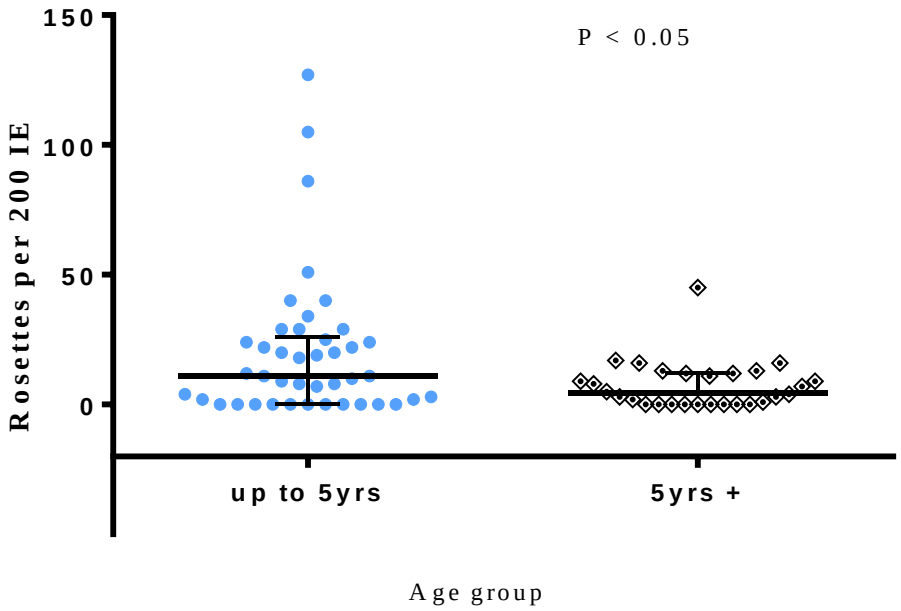


Figure 4.22 **Rosetting rates in two age groups of 0-5 and 5-12 years.** Rosetting rates observed for the 0-5 year lower age group (n= 42) were significantly higher (p = 0.038, Mann-Whitney test) than rates observed in the older 5 years plus age group (n= 28) group. Median values depicted by the long black horizontal bars, along with shorter bars representing the interquartile ranges

4.7 Antibody recognition profiles of patient sera to selected parasites

After studying the wild type clinical parasite isolates, investigations into the immune reactivity of patient sera to the well-characterized A- and B-binding parasites were done. Flow cytometric analysis of patient sera profiles to RBC infected with parasites selected on blood group antigens indicated increased recognition of IE of the selected parasite lines (Figure 4.23). As shown, generally higher mean fluorescence intensities (MFI) were observed for selected parasites BGA7 and BGB7 than for the isogenic, unselected 3D7 strain.

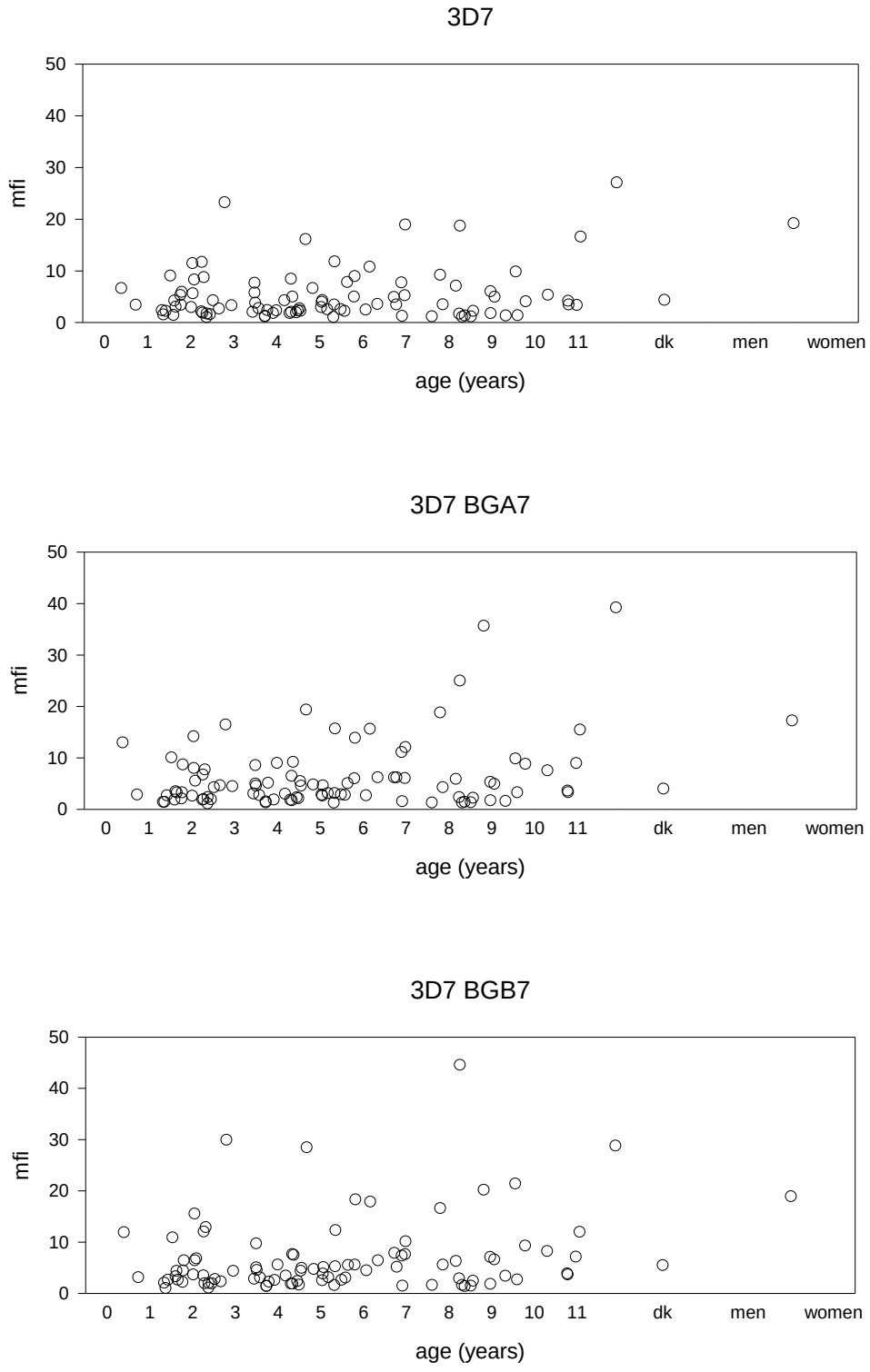


Figure 4.23 **Mean Fluorescence Intensity of bound antibody from donor plasma**. The three panels show MFI values for antibody reactivity to unselected 3D7, A-BSA selected 3D7 BGA7 and B-BSA selected 3D7 BGB7 parasites. Each circle represents an individual donor. dk= pool of malaria naïve Danish plasma

The recognition profile for selected and unselected parasites as measured by the MFI shows differences between 3D7 and FMG parasites (Figure 4.25, 1st and 2nd panels), but no real change in HB3 parasites (Figure 4.24, 3rd panel). Again, the observed change in recognition increased in the older children.

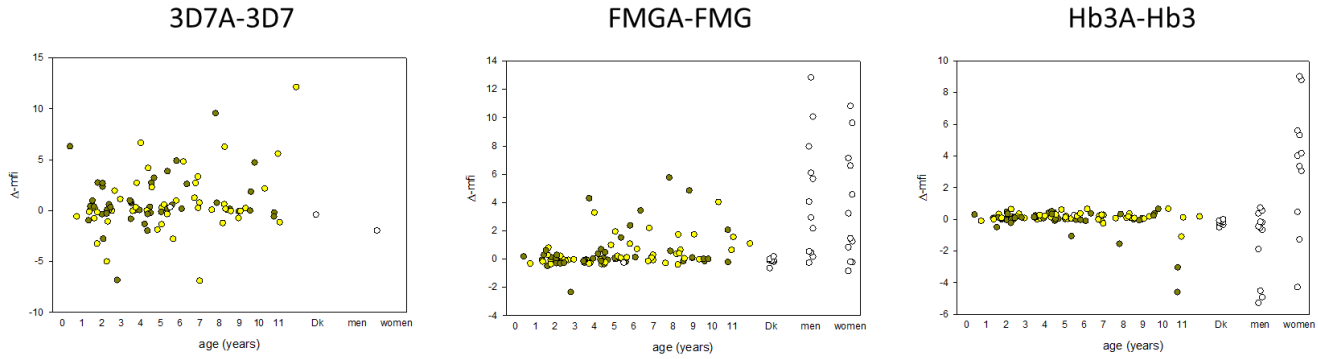


Figure 4.24 Difference in mean fluorescence intensity between selected and unselected 3D7, FMG and HB3 parasites. Generally, Recognition of the IEs by IgG in a pool of plasma from non-exposed donors was always less than 5.5 (3D7) or less than 1.5 (FMG and HB3).

CHAPTER 5

5.0 DISCUSSION

Falciparum malaria pathogenesis is linked to the ability of the parasite to evade host immune responses. Adhesion to the endothelium by mature blood stages of the parasite is critical in this process, enabling the parasite to avoid clearance by the spleen. This may impair circulation in micro vessels and activate ECs, leading to complications and severe disease (Warrell *et al.*, 1988, Moxon *et al.*, 2013). The related rosette formation phenomenon, mediated by exported parasite antigens on the IE surface, is also linked to disease severity (Carlson *et al.*, 1990). One among many (Yam *et al.*, 2017) receptors on RBC for rosetting is blood group A or B glyco-antigens (Carlson and Wahlgren, 1992). The well-established protection against severe falciparum malaria of blood group O individuals (Degarege *et al.*, 2019) has been linked to reduced rosetting (Rowe *et al.*, 2007). Several members of the *var* gene family (Vigan-Womas *et al.*, 2012, Chen *et al.*, 2000b, Angeletti *et al.*, 2012), and most recently members of the *rif* gene family (Goel *et al.*, 2015) have been proposed to encode blood group A (and/or B) adhesion molecules. The parasite strains used in these studies were originally selected for adhesion to whole cells, EC-like or RBC (rosette-selection) or passed through animals. All these procedures are likely to select for adhesion to multiple host receptors.

This study sought to investigate the interaction of IE with ABO blood group antigens directly, and in the absence of other IE or EC receptors for IE adhesion. To achieve this without the effects of other factors, blood group A- and B- specific binding phenotypes were successfully generated by repeated panning of known laboratory strains of *P. falciparum* on immobilized blood group oligosaccharide constructs. The binding patterns of these phenotypes were studied by developing a novel assay to specifically

measure the adhesion of IE to blood group antigens. For these generated phenotypes, a good correlation was found between IE binding to blood group antigens and rosetting in non-O blood group RBCs.

The *in vitro* detection and measurement of host IE interactions are usually done by static, flow-based or cellular adhesion assays (Butler *et al.*, 2016, Adams and Rowe, 2013), rosetting assays or flow cytometric determination of surface markers or rosettes using multiplet discrimination (Ch'ng *et al.*, 2016). The majority of these involve either the subjective and laborious manual counting of Giemsa or fluorescence stained RBC by microscopy, which is the 'gold standard', or the use of specialised or expensive equipment. More recently there have been developments in this area, where assays have been developed to reliably quantify bound parasites using image analysis software without the use of specialized equipment or radioactive labelling (Paton *et al.*, 2011), some requiring just an ELISA reader (Hempel *et al.*, 2015).

A limitation of most of these methods for studies on the interaction between host receptors and the IE is that a range of cell surface receptors may impact this interaction (Andrews *et al.*, 2005). In order to study the isolated effect of ABO blood group binding, a micro titre plate-based assay was developed using a simple protocol and a micro titre plate absorbance reader. The quantification method used here capitalises on the inherent peroxidase activity of RBC (Huy *et al.*, 2005), which has been shown to have the same detection level as currently used methods (Hempel *et al.*, 2015).

The binding and specificity of the commercially obtained glyco-constructs was confirmed in a lectin-binding assay, in which lectin binding can be detected using ELISA-based methods that take advantage of the specific binding between particular lectins and corresponding carbohydrates (Shao, 1992).

The procedure for the blood group binding assay itself was broadly based on the method of Hempel and colleagues to measure cytoadhesion of *P. falciparum* IEs to immobilised ECs (Hempel *et al.*, 2015). In the present study, the three BSA-coupled blood group antigens were incubated overnight in PBS to coat the antigens onto polystyrene micro titre plates before incubation with IE of both selected and unselected laboratory adapted parasites, or with field isolates. Next, a critical gravity washing step was done where the plate was fully submerged and inverted in wash buffer to remove unbound cells from the wells (Paton *et al.*, 2011). Bound RBC were then disrupted to release haem, which acts as a pseudo-peroxidase for oxidation of the TMB chromogenic substrate (Streefkerk, 1972), and the plate subsequently read in a spectrophotometer for quantification using standards included on the plate. In conclusion, this assay was able to detect and quantitatively measure the binding of *P. falciparum* IEs to plate-bound, BSA-coupled oligosaccharides of blood group A, B and O.

Following a number of pannings on ABO oligosaccharide coated wells, it was possible to select parasites with the same blood group-binding phenotype; binding that could be blocked by the relevant selective anti-A and -B antibodies (Figure 4.6). This indicates that the binding was specific for the A- and B-antigens and not due to irrelevant binding to e.g. the plastic surface of the wells or the BSA-anchor. Of concern was a slight difference in construct spacer arm length to the BSA for the O- on one hand, and the non-O blood groups on the other, as these had 3- and 6- spacer arms respectively. However, a comparison of adhesion to blood group B constructs with both 3- and 6-spacer arm lengths showed no difference, for the FMG parasite line, and only a minor difference for the 3D7 line, indicating that this difference in spacer arm length had no significant effect on binding to the constructs.

The biological relevance of the test was indicated by the association between ABO-binding and rosetting. As mentioned in the literature background (Section 2.5.3), the rosetting phenotype is strongly associated with non-O blood groups (Rowe *et al.*, 1995, Carlson *et al.*, 1994, Barragan *et al.*, 2000). Thus, the 3D7, FMG and FUP parasites selected on blood group A or B antigens spontaneously formed rosettes in non-group O RBC, unlike their parental unselected lines that did not form rosettes in any blood type.

The assay has the added advantage of being easy to execute, with higher throughput and limited requirement for reagents and equipment. As with other static assays however, this assay may be less representative of an *in vivo* physiological environment when compared to a flow-based assay. In the present study the focus was not to try to replicate an *in vivo* environment, where binding characteristics may be different or change altogether (Adams *et al.*, 2000, Crabb *et al.*, 1997). It was primarily to eliminate exogenous factors in investigating IE/ABO-blood group antigen interactions. The previously mentioned alternative methods, though fairly accurate, nonetheless require expensive equipment or reagents, specialist training and, especially under field conditions, may be impractical in some cases.

In summary, this new assay to measure IE binding to ABO blood group antigens offers a fast and objective measure of the binding of IE to multiple blood group antigens within the same assay. This novel assay is able to discriminate between binding phenotypes and also correlates satisfactorily with IE preference for rosetting in blood group A, B and AB, making it a useful alternative to the subjective counting of rosettes by microscopy, particularly in instances where RBC numbers in large rosettes are difficult to determine.

Panning on either blood group A or B BSA-coupled oligosaccharides adsorbed onto culture dishes was able to induce successful selection of A- and B-binding phenotypes

in some but not all the tested culture-adapted laboratory isolates of *P. falciparum*. This procedure yielded blood group A and B binding sublines for 3D7, FMG and FUP, with several times (up to 7 fold) higher binding than the unselected parent lines. In contrast, no A- or B-binding could be induced with the isolates HB3 or FCR3, despite several attempts of repeated panning. This may be due in part to the fact that there are differences in binding preference of some isolates between surface expressed proteins on IE and immobilized antigens in a non-physiological environment (Azasi *et al.*, 2018), or that the parasites in question simply lack the relevant VSA genes, or have them 'switched off'.

Interestingly, parasites selected for binding to blood group A antigens also bound blood group B antigens, as did the blood group B selected parasites, which bound blood group A sugars. Also, rosetting in non-group O RBC could be induced by panning on either A- or B-oligosaccharides, an indication that these parasites may share a common ligand that mediates blood group A or B binding to IE. This may be a reflection of the structural similarity between these two blood types, where the only difference is the addition (to the base H antigen oligosaccharide) of *N*-acetylgalactosamine (GalNAc) for blood type A or galactose (Gal) for blood type B.

In summary, specific adhesion phenotypes of rosetting isolates that bind to blood group polysaccharides have been successfully established for both 3D7 and FMG strains of *Plasmodium*. These isolates, which are stable and maintain the phenotype over several months, would be useful for studies into, and comparison of blood group adhesion phenotypes and *in vitro* studies of adhesion-related manifestations of the disease.

The binding characteristics of RBC infected with late stages of the 3D7 and FMG strains that were successfully selected for binding to blood group A and B, along with the HB3 strain for which selection was not possible, were tested on human aorta and

dermal ECs to investigate cytoadhesion in these parasites. The selected 3D7 and FMG strains showed higher binding rates than their unselected parental strains.

The ABO blood group antigens are known to be expressed on both RBCs and other tissues, including the endothelium (Wang *et al.*, 2013), and also on all but one vascular EC line (O'Donnell *et al.*, 2000). Endothelial cell binding assays reflect the *in vivo* property of sequestration by late stage parasites to avoid splenic clearance (Udeinya *et al.*, 1981, Utter *et al.*, 2017). The differential phenotype of higher binding observed in selected 3D7 and FMG parasites in this study suggests a strong link between binding to A- and B- antigens and adhesion to ECs. It could be inferred here that circulating parasites with this phenotype could have a better chance of survival *in vivo* through sequestration and immune evasion (Ockenhouse *et al.*, 1991). The rosetting and cyto-adhesive phenotype shared by all successfully selected parasites in the current study strengthens the notion that these two *attributes* are linked, mediated by the parasite-derived variant surface antigens PfEMP1 (Rowe *et al.*, 2009) and/or RIFINs (Goel *et al.*, 2015), and dependent on blood type. Some studies on the other hand have identified distinct receptor-ligand interactions for both rosetting and cytoadhesion in the same parasite via separate PfEMP1 domains (Adams *et al.*, 2014), indicating that rosetting is distinct from cytoadhesion.

In the case of HB3, for which selection for binding to the A and B constructs was not possible, a different picture also emerged with regards to EC adhesion. Unlike for 3D7 and FMG, the unselected HB3 bound to EC before selection, indicating that this parasite was able to adhere to EC by an adhesin that does not bind blood group A or B presented on BSA constructs. Selection on blood group B did not induce any detectable changes in adhesive behavior. The A-selected HB3, however displayed a very high degree of binding to cell-culture wells without EC, probably reflecting that in this case a VSA

with high affinity for the culture-dishes or BSA rather than the A-oligosaccharide had been selected for by panning. It is noteworthy that the selection was done in 24-well culture plates made of the same material as the 96 well plates used for EC adhesion assay, whereas the direct binding to A and B oligo saccharide constructs was performed in 96-well 'Polysorb' plates. The apparent 'accidental' selection for a parasite with a high capacity for binding to empty cell culture dishes highlight the importance of validating the specific adhesion phenotypes carefully, particularly in studies where parasites are manipulated *in vitro* to bind to various receptors found on EC or syncytiotrophoblast.

In all, the blood group A and B binding phenotypes produced by selection on blood group antigens show a clear preference for rosetting in RBC from non-group O donors. As indicated earlier (Figure 4.10), there is a good correlation between the rosetting and binding assay results for the selected phenotypes, and could serve as a good correlate of rosetting and in turn, sequestration, as rosetting is a phenotype known to be associated with severe malaria (Carlson, 1993, Carlson *et al.*, 1990, Rowe *et al.*, 1995, Doumbo *et al.*, 2009). This could improve the study of malaria and blood group linked studies on rosetting and binding phenotypes in both laboratory and field based studies.

To detect potential VSAs of interest that may be involved in the blood group antigen binding phenotypes, the 3D7 and FMG parasite strains selected on blood group A and B antigens were analysed for their gene expression profiles by QPCR, under the assumption that these phenotypes are attributable to particular *var* or *rif* genes, and that expression can be correlated to increased mRNA levels.

The changes observed in the phenotypes of parasites, including their cytoadhesion characteristics, have often been investigated by analysing the *var* gene expression profiles of these parasites (Smith *et al.*, 1995, Golnitz *et al.*, 2008, Salanti *et al.*, 2003,

Albrecht *et al.*, 2011). In the current study, expression profiles of 58 *var* and 158 *rif* genes in selected and unselected 3D7 parasites were initially determined. From the analysis of this data, only one *var* gene previously associated with blood group A mediated rosetting, Pf13_0003, appeared to have slightly increased transcription levels as a consequence of blood group A and B selection (with fold changes of 1.3 and 1.7 respectively). All other *var* genes were transcribed at a lower level in the selected 3D7 parasites, resulting in an overall 3- fold decrease in total *var* transcription. Similarly, no transcriptional changes in *rif* genes previously associated with preferential rosetting in blood group A or a change in the balance between group A and B *rif* genes could be detected.

Pf13_0003 is known to encode the 3D7 orthologue of adhesion domains that mediate rosetting (Bull *et al.*, 2005, Gardner *et al.*, 2002), and it would appear that Pf13_0003, and therefore DC5 *var* genes (and/or SD3-loop containing *var* genes) mediate the interaction observed in the isolates from the present study (Angeletti *et al.*, 2012). To validate these findings, the analysis was repeated for unselected 3D7 and blood group A selected 3D7. In this second experiment, the difference in transcription of Pf13_0003 between blood group A selected and unselected 3D7 was more pronounced (Table 4.2). Comparing the results of the second QPCR experiment with the first however, it appears the observed effect resulted from a decrease in transcription that had happened during the time between the two experiments in the unselected 3D7, while the expression of Pf13_0003 remained at approximately the same level in the blood group A selected substrain. The total decrease in *var* transcription was less pronounced in the second experiment with 3D7. This was mainly due to increased transcription of group B *var* genes not previously associated with rosetting.

Though the initial QPCR experiments with the 3D7 parasite lines did not reveal consistent upregulation of any *var* gene, it appeared that DC5 or SD3 loop containing *var* genes such as PF13_0003 would be the most likely candidates mediating blood group A and B adhesion among those candidates already suggested. This necessitated a look at the closely related *var* genes of the blood group selected FMG parasite line for similar trends in transcription. Thus, transcription of 56 *var* genes, including the DC5 *var* gene IT4var2, the SD3 containing IT4var9 (R29var) and IT4var60 as well as IT4var21 that has also been implicated in rosetting (Vigan-Womas *et al.*, 2008, Adams *et al.*, 2014) were analysed by QPCR. Results from analysis of the transcript levels turned out not to be in agreement with that for 3D7, as both the SD3 loop containing *var* genes as well as IT4var21 and IT4var2 showed overall very low transcription in all 3 FMG lines (less than 1% of all *var* transcripts in all parasites), with decreased transcription in selected relative to the unselected control for all but the DC5 *var* gene IT4var2 that was transcribed at the same level in all parasites (fold changes 1.2 and 1.0 in the A and B selected FMG parasites, respectively). The lone *var* transcript for the FMG selected parasites showing an elevated transcript level in the selected lines compared to the parental line was IT4var 32b (Fold changes of 6.7 and 1.3 in the A and B selected lines respectively (Figure 4.13). This *var* gene belongs to the DC8 *var* gene group, responsible for binding to EPCR, and has not previously been suggested to mediate rosetting via blood group A. This finding was in contrast to the profile observed for the 3D7 isolate, where two DC8 *var* genes were downregulated 3 to 10-fold in selected parasites. These results, due to the differences in transcript changes, may point to the fact that the adhesion phenotype selected for is not linked to a particular *var* gene expression, and that the observed upregulation of Pf13_0003 in 3D7 and IT4var32b in FMG could be explained as random events. Alternatively, the phenotype may be independent from DC structure and be mediated by a yet unidentified motif shared

between PF13_0003 of 3D7 and IT4var32b. With the limitation that not all IT4 *var* genes were included in the FMG analysis, the existing data set is consistent with an overall down regulation of *var* genes as the main trend in blood group A and B selected parasites. This could be an artefact appearing from an increased transcription of the housekeeping genes used for normalisation of *var* gene transcription that had arisen from the selection procedure. The number of housekeeping transcripts detected in each different parasite sub line tested in the same experiments were more similar than the total number of *var* transcripts detected, and the relative abundance of the 2 housekeeping genes was similar, indicating that the transcription of the housekeeping gene was similar in selected and unselected parasites.

In the first QPCR experiment with 3D7, transcription of *rif* genes were also slightly decreased in selected parasites relative to the unselected, and there was no prominent or specific transcription that was consistent in both the A and the B selected line linked to the phenotype under observation. In the second experiment one *rif* gene, Pf13_0004 appeared to be very prominently transcribed in the blood group A selected 3D7 relative to unselected 3D7. This however was not seen in the first experiment where the phenotype in question had already been acquired. No major changes in the balance between group A and B RIFINs were detected, and the *rif* gene previously described to mediate blood group A dependent rosetting by Goel *et al.* (2015), PFA0020w, was transcribed at a very low level in both selected and unselected parasites. The data presented here are not consistent with group A RIFINs in general or certain RIFINs being the main blood group A and B binding adhesins.

This may highlight the limitations of the method used here, since the transcription profiles of the *var* and *rif* genes of these parasites, investigated using QPCR, only offers a limited look at the broader aspect which can be achieved by other approaches such as

RNA-seq or gene arrays to identify novel transcripts as well as differentially expressed genes (Tarr *et al.*, 2018).

This study has successfully developed blood group binding isolates and answers basic questions on transcripts that may be linked to the observed phenotype, and paves the way for further molecular investigations into understanding interactions between parasite and host in *falciparum* malaria.

The distribution of blood group A, B, AB and O frequencies across the sampled patients were respectively 21%, 24%, 4% and 51%, generally similar to frequencies reported in other studies in Ghana (Acquaye, 2004) and in the sub region (Tadesse and Tadesse, 2013, Zerihun *et al.*, 2011). The higher frequencies of blood group O is not unexpected, as this phenotype is known to protect from severe disease (Panda *et al.*, 2011, Degarege *et al.*, 2019), and is indeed highest in malaria endemic regions worldwide (Cserti and Dzik, 2007).

A total of 39 clinical *P. falciparum* isolates were tested for their ability to bind the blood group antigens. Eight of these isolates showed binding to at least one blood group construct. Of these, 5 were from patients of blood type O, 2 from blood type B and 1 from blood type A.

Generally, as observed for the laboratory strains, most field isolates that did show binding, bound to both the A and B constructs. Binding to the H antigen construct (group O antigen) by field isolates was virtually absent and only noted in one borderline case, which interestingly also bound the A and B blood group constructs.

Another observation made about the 8 blood group A and B binding cohort from the field isolates is that all the donors of the binding parasites were below the age of 5 years, the age most at risk of severe disease. Considering that immunity to malaria

occurs over many years, and the fact that this immunity is first acquired to the most virulent strains (Gupta *et al.*, 1999), it is understandable that isolates that bind blood group antigens (and therefore most likely to form rosettes or adhere to ECs, leading to severe disease) are found in children under 5 years who are unlikely to have a full repertoire of protective antibodies.

No difference was observed in the binding capacity of IE to the blood group A antigen, irrespective of whether the donor was blood group O or a non-group O patient. In other words, the ability to bind to the A antigen was independent of donor blood type. Therefore, blood group A and B-binding parasites can be found in hosts with blood group O as well. It is therefore possible that the blood group A and B adhesin may bind other host receptors in addition to the blood group determinants such as PECAM1 (CD31) or von Willebrand factor, which are known to carry ABH determinants. It is noteworthy that the DC5 group A *var* genes, which contain PF13_0003, have previously been implicated in PECAM1 mediated endothelial adhesion (Berger *et al.*, 2013).

Rosetting assays were carried out in 70 clinical isolates, for 50 of which at least one rosette was observed. The distribution of rosettes observed between the various blood groups in this study were not statistically different, though it is worth mentioning that the mean rosetting rates observed for non-group O patients was about twice that observed for blood group O patients. Previous studies including cohorts with larger numbers and younger patients however showed significantly lower rosetting rates in blood group O than non-O patients (Rowe *et al.*, 1995, Udomsangpetch *et al.*, 1993). In the present study, rosetting rates in children 5 years or less were significantly higher than their older counterparts. The elevated rosetting rates in young children could be

attributed to the non-group O blood groups, since there was no decline in rosetting with age for blood type O patients.

To test if blood group A- and B-binding parasites were most commonly encountered by patients with non-O blood type, investigations into the ability of patient antibody to recognise parasites selected for binding to blood group antigens was determined by flow cytometry. Selected and unselected parasites of 3D7, FMG and HB3 strains were incubated with sera from the study cohort.

Overall recognition of selected parasites by patient antibody was very similar to their unselected parental parasite line. This is a very different scenario from the dramatic change in serological recognition previously described for pregnant women following selection for binding to CSA (Sharling *et al.*, 2004, Salanti *et al.*, 2004). However, detailed analysis subtracting the MFI values obtained for the unselected parasite from the MFI value obtained for the selected parasite revealed that some individuals recognised the selected parasites slightly better than the unselected parasite. This increased recognition of selected parasites was found to increase with age, with older children recording higher MFI shifts compared to younger children. There was no difference between plasma donors of non-O and O blood types with regard to MFI shift in selected parasites. This is in concordance with the observation that blood group A and B adhesion was found in type O parasite donors as well as in non-O donors. This observation was made in both 3D7 and FMG isolates, though not for the HB3 isolate, where, as expected, no difference in recognition between selected and unselected parasites was observed in plasma from children.

The overall very subtle change in recognition pattern following selection for binding to blood group A and B is in concordance with the finding that no major changes in the

composition of the major var and rif VSA families that have been shown to be the main targets of naturally acquired IE antibodies, were detected in the QPCR analysis.

The findings accrued from this study could impact interventions in several ways. For example the binding assay provides an alternative procedure to quantify adhesion to blood group polysaccharides (or other ligands) without radioactive labelling of parasites or laborious error prone counting by microscopy. This information could be relevant in a clinical setting where parasite binding preference could predict or confirm severe disease such as CM and inform preventive treatment and management. Also, identification of specific parasite VSA epitopes that bind blood group antigens could lead to tailored anti-severe malaria vaccine candidates for vulnerable groups.

5.1 Conclusions and recommendations

5.1.1 Conclusions

In conclusion, the data presented here demonstrates that *P. falciparum* IE adhesion to blood group A and B antigens can be measured directly in a simple plate-based assay using oligosaccharides coupled to BSA. The binding measured by this assay correlates well with preference for rosette formation with RBC of non-O blood types, a phenotype that is associated with severe disease (Rowe *et al.*, 2007). The A/B adhesive IE phenotype was detected by this assay in patient isolates obtained from Ghanaian children under 5 years of age. It was also demonstrated that the blood group A/B adhesion phenotype is linked with avid endothelial adhesion. Altogether these clinical and in vitro observations suggest and support the notion that the A/B adhesive phenotype is associated with parasite virulence. Surprisingly, the recognition patterns of plasma obtained from semi-immune Ghanaian children were very similar for isogenic parasites regardless of whether they would bind strongly to blood group A/B polysaccharides or not. This could indicate that the parasite adhesin selected for by panning on A/B oligosaccharides constitute only a minor part of all the VSAs expressed on *P. falciparum* IEs, or that the adhesin responsible for A/B binding might be of relatively low immunogenicity. The unexpected lack of any major changes in serotype, however, was mirrored by the lack of overall major and consistent changes in the composition of transcribed *var* and *rif* genes before and after selection on ABO blood group oligo saccharides. The most consistent change observed was an overall decrease in *var* gene transcription in the blood group A and B adhesive parasites compared to their unselected parental lines. In summary, the real time quantitative PCR data presented in this work are not in concordance with either a certain sub type of group A-

var genes, the DC5 type, or SD loop containing, or type A *rif* genes being responsible for blood group A/B mediated rosetting.

5.1.2 Recommendations

Two major recommendations for future work have emerged from this project. Firstly, the study of binding to A/B oligosaccharides in patient isolates should be expanded to validate the association between binding to the A/B antigens, preference for rosette formation in non-O RBC and EC adhesion found in patient isolates. Furthermore, the larger group of patients should include well defined severe cases to evaluate whether the A/B adhesive phenotype is indeed associated with increased virulence. The other major recommendation is to broaden the search for the parasite adhesin binding blood group A/B beyond *var* and *rif* genes. As both the 3D7 parasite clone and the FMG strain of It4 genotype have fully sequenced genomes, these parasites could be used in whole genome transcriptional studies as well as in proteomic studies searching for genes differentially expressed between the A/B selected parasites and the unselected lines. For transcriptional analysis RNA sequencing we propose to use mRNA sequencing, as this method, unlike array-based methods, does not depend on exact knowledge of gene sequences. Thus, this approach might also be employed in studies of field isolates with different degrees of blood group A/B adhesion capacity.

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