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Plasmodium falciparum binding interactions with human brain endothelial cells

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Lay Summary

Cerebral malaria, the most severe form of malaria, causes impaired consciousness and coma, with often life-threatening consequences in affected individuals. The disease, which mostly affects children under 5 years, accounts for the majority of the 438,000 annual malaria deaths, and is caused by the malaria parasite called *Plasmodium falciparum*. The parasites invade red blood cells and express a protein on the surface of the red blood cells, which enables them to bind to cells lining the walls of small blood vessels in the brain called endothelial cells. This binding prevents the parasites from being sent to the spleen for destruction, but can cause disease by obstructing blood flow in the brain blood vessels. Parasites that bind to brain endothelial cells express two main types of infected red cell surface protein, which are associated with cerebral malaria. These surface proteins are suggested to bind to molecules on brain endothelial cells called Endothelial Protein C Receptor (EPCR). Knowledge of how this binding occurs might enable the development of therapies for cerebral malaria that target this binding phenomenon to supplement current parasite killing drugs.

Using several different parasites lines, I investigated the environmental conditions of the parasites and brain endothelial cells that may affect the binding. The infected red cell surface proteins expressed by two Kenyan parasites recently adapted to culture that bind to brain endothelial cells and the role of EPCR in the parasite binding were also investigated.

My results showed that the binding depended on pH and the number of parasites (parasitaemia), with the greatest binding occurring at pH7.3 and at high parasitaemia. Binding was not affected by a high temperature of 39°C or low oxygen conditions. Binding of parasites that expressed one of the major types of the surface protein was blocked by human serum suggesting that such parasites may not bind in real life conditions where there is serum in blood. The two Kenyan parasite lines that bound to brain endothelial cells expressed similar types of the infected red cell surface protein as those described previously and also expressed another type of the surface proteins. Binding to

EPCR on the brain endothelial cells was, however, not observed in all the parasites. Parasites that expressed one of the major types of the surface protein (whose binding was blocked by serum) bound to EPCR, while those that expressed the other major surface protein type did not bind to EPCR.

This study adds to current knowledge by highlighting the factors that can affect malaria parasite binding to brain endothelial cells. The findings suggest that parasite expression of the two major types of protein which are associated with cerebral malaria may have different functions; with one contributing to the disease through binding to brain endothelial cells and the other type of surface protein (whose binding is blocked by serum) through a different mechanism yet to be identified. The study also shows that additional receptors, other than EPCR, are required for binding to brain endothelial cells in cerebral malaria. Full understanding of the mechanism of parasite binding is needed in order to develop new treatments that target parasite binding to brain endothelial cells in cerebral malaria.

Abstract

Cerebral malaria is the most severe form of malaria and mostly affects children under 5 years causing impaired consciousness, coma and neurological disorders, with life-threatening consequences in affected individuals. A pathological feature of the disease is the sequestration of mature *Plasmodium falciparum* infected erythrocytes (IEs) in the microvasculature of the brain. *P. falciparum* Erythrocyte Membrane Protein 1 (PfEMP1) expressed on the surface of IEs is thought to enable the parasites bind to human brain endothelial cells (HBEC) to avoid splenic clearance. An *in vitro* model of cytoadherence in cerebral malaria has been developed using a human brain endothelial cell line called HBEC-5i, which enables the study of IEs binding to HBEC.

Previous work based on three laboratory parasite lines showed that HBEC-binding IEs express a specific subset of the diverse PfEMP1 family which contain sets of cysteine-rich domains called domain cassettes (DC) 8 and 13. Parasites from children diagnosed with cerebral malaria have also been independently shown to express the DC8 and DC13 PfEMP1 types. The adhesion of IEs to HBEC is suggested to occur by binding of a domain of the DC8 and DC13 PfEMP1 variants called CIDRα1, to Endothelial Protein C Receptor (EPCR) on HBEC. However, investigations of the effect of parasite and host environmental factors on adhesion to HBEC are lacking, and further studies are needed to confirm the association of these DC8 and DC13 PfEMP1 to HBEC-binding and the role of EPCR in mediating the cytoadhesion. Therefore, the aim of this thesis was to examine the hypothesis that HBEC-binding would be affected by changes in environmental conditions, and that all IEs that bind to HBEC would express group A-like PfEMP1 containing DC8 and DC13, for binding to EPCR on HBEC.

In this study, the effect of pH, parasitaemia, gas, temperature, and serum on cytoadhesion, were investigated using four HBEC-binding parasite lines. Adhesion of IEs to HBEC was found to be pH and parasitaemia -dependent with optimal binding at pH 7.3 and IE adhesion positively correlated with parasitaemia. There was no significant effect of increase in temperature to 39°C and no significant difference between hypoxic and normoxic conditions on adhesion in all parasite lines. Human serum, however, abolished binding of the DC8-expressing parasite line but had minimal effects on adhesion of the DC13-expressing parasite lines.

Two Kenyan isolates recently adapted to culture were selected for binding to HBEC and were found to also predominantly express group A-like PfEMP1 including a DC8 PfEMP1 variant and PfEMP1 (s) that contained DBLα1.2 domains. Attempts were made to localise the binding domain within the DC8 and DC13 PfEMP1 variants using recombinant proteins and antibodies. However, the CIDRα1 domain appeared to mediate adhesion of the DC8-expressing parasite line but had no effect on adhesion of the DC13-expressing parasite lines. Only antibodies to the N-terminal domain, known as NTS.DBLα, significantly inhibited binding of all the parasites lines.

The role of EPCR and other receptor molecules on endothelial cells including ICAM-1, CD36, CSA, PECAM-1, HABP-1 and heparin, in mediating adhesion to HBEC was also investigated. Using EPCR recombinant protein, monoclonal and polyclonal antibodies, and EPCR-siRNA knockdown in binding assays, EPCR was shown to be involved in adhesion of only the DC8-expressing parasite line and did not affect adhesion of the DC13-expressing parasite lines to HBEC. Binding of DC8-expressing parasite line to HBEC was also inhibited by soluble recombinant PECAM-1. There was no significant adhesion of both types of parasite lines to the other receptor molecules, although minimal binding to HABP-1 was observed.

This study expands current knowledge on the parasite binding interactions with HBEC by elucidating some of the environmental factors that affect the binding properties, and gives the optimal conditions for the *in vitro* model of HBEC-adhesion in cerebral malaria. Findings presented here confirm the association of expression of group A-like PfEMP1 to HBEC-binding and shows that the EPCR-CIDRα1 interaction does not mediate adhesion of all DC8 and DC13- expressing parasite lines to HBEC. Additional receptors, other than EPCR, are therefore required for HBEC-binding in cerebral malaria. The ability of normal human serum to abolish binding of the DC8-expressing parasite line also raises the question of whether IE binding to EPCR is physiologically relevant and suggest that the DC8-expressing parasites associated with cerebral malaria may contribute to the disease in a mechanism other than binding to brain endothelial cells.

Declaration

I declare that the content of this thesis is my own work and has not been done in collaboration, except where otherwise stated. No material presented in thesis has been submitted to any other university or for any other degree.

Yvonne Azasi

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Finally, all thanksgiving and praise be to God for…everything!

Abbreviations

°C	Degree Celsius
ACD	Acid citrate dextrose
ANOVA	Analysis of variance
APC	Activated Protein C
ATS	Acidic terminal segment
BSA	Bovine serum albumin
DABCO	1,4-Diazabicyclo[2.2.2]octane
DC	Domain cassette
CD36	Cluster of differentiation 36
cDNA	Complementary DNA
CIDR	Cysteine-rich interdomain region
CSA	Chondroitin sulfate A
DAPI	4,6-diamidino-2-phenylindole
DBL	Duffy-like binding domain
ddH_2O	Double distilled water
DEPC	Diethylpyrocarbonate
dH_2O	Distilled water
DMEM	Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 Ham
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
EPCR	Endothelial Protein C Receptor
FACS	Fluorescent activated cell sorting
FBS	Foetal bovine serum
FITC	Fluorescein isothiocyanate
gDNA	Genomic DNA
HABP-1	Hyaluronan binding protein 1
HBEC	Human brain endothelial cells

HBMEC	Human brain microvascular endothelial cells
HEPES	4-(2-hydroxyethyl)-piperazine-ethanesulfonic acid
HI	Heat inactivated
ICAM-1	Intercellular adhesion molecule 1
IE	Infected erythrocytes
IFA	Immunoflourescence assay
IFN	Interferon
IL	Interleukin
IPTG	Isopropyl β-D-1-thiogalactopyranoside
KAHRP	Knob-associated histidine-rich protein
LB	Luria-Bertani
MACS	Magnetic-activated cell sorting
MESA	Mature parasite-infected erythrocyte surface antigen
NCAM-1	Neural cell adhesion molecule 1
NTS	N-terminal segment
OPD	o-Phenylenediamine dihydrochloride
PAM	Pregnancy associated malaria
PAR1	Protease-activated receptor 1
PBS	Phosphate-buffered saline
PBST	Phosphate-buffered saline Tween
PCR	Polymerase chain reaction
pcv	Packed cell volume
PECAM-1	Platelet endothelial cell adhesion molecule 1
PfEMP1	*P. falciparum* erythrocyte membrane protein one
PfMC-2TM	*P. falciparum* Maurer's cleft two transmembrane protein
PMR	Parasite multiplication rates
Rif / RIFIN	Repetitive interspersed family
RPMI	Roswell Park Memorial Institute
RT-PCR	Reverse transcriptase polymerase chain reaction
RNA	Ribonucleic acid
SDS	Sodium dodecyl sulfate
SEM	Standard error of mean

STEVOR	Subtelomeric variable open reading frame
TEER	Trans-endothelial electrical resistance
THBMEC	Transformed human brain microvascular endothelial cells
TNF	Tumor necrosis factor
Ups	Upstream sequence
VCAM-1	Vascular cell adhesion protein 1
VSA	Variant surface antigen
WHO	World Health Organisation

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1 Chapter I: Introduction

A general introduction to malaria and the thesis topic is shown here. A specific introduction is also given with each results chapter.

1.1 Malaria - a general background

Malaria burden remains high with more than 40% of the world's population at risk of the disease and estimated annual cases over 200 million. Despite the decline in the number of malaria cases and associated deaths, the disease causes more than 400,000 deaths annually mostly in sub-Saharan Africa (WHO, 2015) .

Malaria is an infectious disease caused by the apicomplexan, *Plasmodium*. In humans, malaria can be caused by *P. falciparum, P. vivax, P. ovale, P. malariae* or *P. knowlesi* (infects humans by zoonosis (Singh and Daneshvar, 2013)). *P. falciparum,* which is prevalent in sub-Saharan Africa, causes the most life threating malaria disease, and is responsible for most malaria deaths. *P. vivax* which is prevalent in Asia, although it originated from Africa (Liu *et al.*, 2014) could also cause severe disease (Manning *et al.*, 2011; Rahimi *et al.*, 2014; Barber *et al.*, 2015) although this remains controversial.

Due to advancement in treatment and preventive measures, malaria control programmes have been successful at reducing the transmission and mortality (Bhatt *et al.*, 2015). Recent emergence of insecticide resistance and resistance to artemisinin, the most potent drug for treatment of malaria, possess a threat to recent progress in malaria control (Phyo *et al.*, 2012; Ashley *et al.*, 2014).

1.1.1 The life cycle

Malaria is a vector-borne disease that is transmitted by *Plasmodium*-infected female *Anopheles* mosquitoes. The mosquito is the obligate vector required for sexual development of the parasite.

An infected mosquito transmits the parasites by injecting them in the form of sporozoites in the salivary glands, into the skin of the host during a blood meal (Figure 1.1). The sporozoites move through the blood stream to the liver, then through Kupffer cells into hepatocytes where they develop and replicate by mitotic divisions (within 5 to 15 days depending on the species) to form schizonts which contain thousands of merozoites (Prudêncio, Rodriguez and Mota, 2006). In the case of *P. vivax* and *P. ovale*, some of the sporozoites in the liver form hypnozoites that can remain dormant in the hepatocytes for several weeks to years and are activated by an unknown mechanism to resume replication (Shanks and White, 2013). The merozoites are released into the bloodstream and invade erythrocytes to begin the asexual intra-erythrocytic stage. In the infected erythrocyte, the merozoites form rings that mature into pigmented trophozoites, which undergo schizogony to form schizonts. Merozoites released from the schizonts, go on to invade erythrocytes and continue the erythrocytic cycle. Some of the parasites from the schizonts differentiate into the sexual forms of the parasites; male and female gametocytes which are ingested during a blood meal by a mosquito (Silvestrini, Alano and Williams, 2000). The male and female gametocytes are activated to form gametes that fuse in the midgut of the mosquito to form a zygote. The zygote develops into an ookinete and the motile ookinete transverses the epithelial wall of the midgut to form an oocyst. The oocyst undergoes several rounds of mitosis to produce thousands of sporozoites that migrate to the salivary glands of the mosquito (Gerald, Mahajan and Kumar, 2011). The sporozoites are then injected into the host when feeding and the cycle continues.

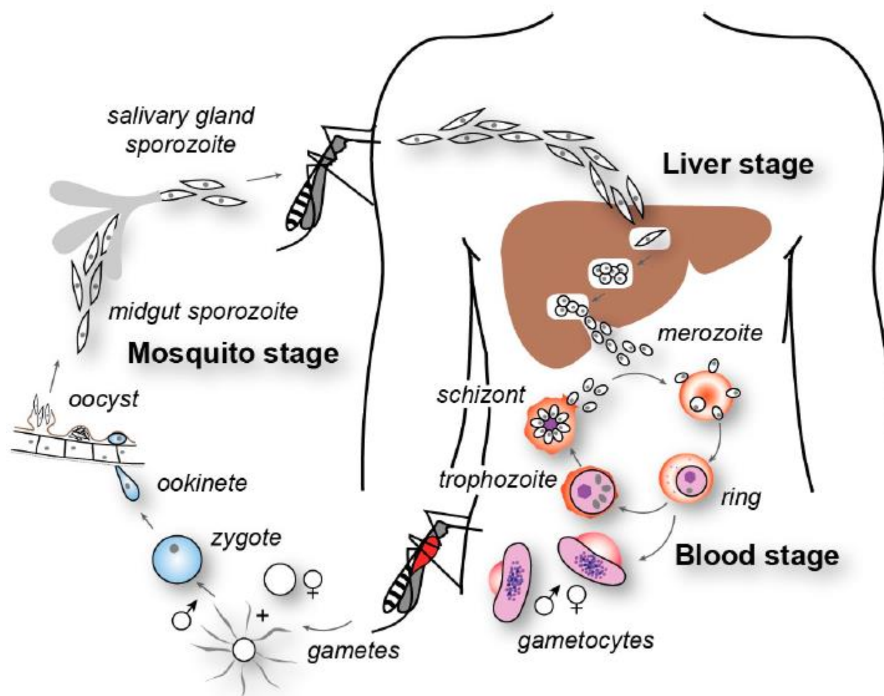


Figure 1.1. The life cycle of *Plasmodium*. An illustration of the various developmental stages (from infection to transmission) the *Plasmodium* parasites undergo from the liver and the blood stream of the host and in the gut of the *Anopheles* mosquito. The figure was taken directly from Cowman *et al.* (Cowman, Berry and Baum, 2012).

1.1.2 Pathogenesis of *P. falciparum* malaria

Malaria is diagnosed by the detection of *Plasmodium* with microscopy or antigen-based rapid diagnostic tests. There are two types of presentation of the disease: mild or uncomplicated malaria and severe malaria. There are, however, individuals with asymptomatic infections who do not show signs of the disease.

Symptoms of the disease, which include fever, chills, headaches, nausea, vomiting, malaise and lethargy, occur during the erythrocytic stage at about 7 to 14 days after infection. These symptoms are usually characteristic of uncomplicated malaria. Clinical features of severe malaria, however, include metabolic acidosis, respiratory distress, prostration, severe anaemia (haemoglobin concentration below 5 g/dl), repeated convulsions, hypoglycaemia (blood glucose below 40 mg/dl or 2.2 mmol/l) and coma (WHO,

2014). Metabolic acidosis and respiratory distress are features of severe malaria strongly associated with fatal outcome (English *et al.*, 1996; MacKintosh, Beeson and Marsh, 2004; Maitland and Marsh, 2004). The release of parasite toxins into the bloodstream, destruction of IEs, host immune response and sequestration of IEs contributes to the pathology of the disease.

Parasite infection of erythrocytes leads to deformation of the infected erythrocytes (IEs), which results in their destruction. Uninfected erythrocytes are also lost by complement-mediate lysis and phagocytosis by monocytes and macrophages (reviewed in MacKintosh, Beeson and Marsh, 2004; Milner *et al.*, 2008). Erythropoiesis is also impaired during malaria infection and this has been suggested to be due to inflammatory mediators such as cytokines (Haldar and Mohandas, 2009). This increased loss of erythrocytes can result in severe anaemia in affected individuals.

Immune inflammatory response is associated with some of the clinical features of the disease. High levels of pro-inflammatory cytokines including tumor necrosis factor alpha (TNF-α), Interferon gamma (IFN-γ), Interleukin (IL) -1β IL-2, IL-6, IL-8, IL-10 and IL-12 have been reported in both children and adults with symptomatic *P. falciparum* malaria compared to asymptomatic or healthy controls (Day *et al.*, 1999; Lyke *et al.*, 2004; Walther *et al.*, 2006; Mirghani *et al.*, 2011). The role of inflammatory response in severe malaria is however not clear, as higher levels of the cytokines were observed in non-severe *P. vivax* infections (Zeyrek *et al.*, 2006; Jain *et al.*, 2010; Gonçalves *et al.*, 2012; Scherer *et al.*, 2016) compared to *P. falciparum* infections. It has been suggested that control of inflammatory response early in life may protect children, who are yet to develop antibodies to *P. falciparum* antigens, against severe malaria (Molineaux *et al.*, 2002; Portugal, Pierce and Crompton, 2013).

Parasite binding interactions also contribute to the pathology of the disease. The ability of IEs to bind to host cells in the microvasculature to avoid splenic clearance, causes obstruction of blood flow (Dondorp *et al.*, 2008). This sequestration of IEs in microvasculature results in impaired tissue perfusion due to the binding of IEs to the endothelium, congestion of infected and uninfected erythrocytes and reduction in microcirculatory blood flow, and has

been associated with metabolic acidosis and coma in individuals with cerebral malaria (reviewed in Miller *et al.*, 2002; Dondorp, Pongponratn and White, 2004; Ponsford *et al.*, 2012).

Individuals who live in malaria endemic regions (88% in sub-Saharan Africa where *P. falciparum* is prevalent (WHO, 2015)) develop partial immunity to severe malaria but remain susceptible to asymptomatic infections (reviewed in Hviid 2005). A study that looked at various indicators of immune response including opsonisation of IEs, showed that antibodies to the variant surface antigens (VSA) on the IEs was the only factor that correlated with protection against severe disease (Marsh *et al.*, 1989). Healthy individuals (who have had previous episodes of malaria), asymptomatic individuals and those recovering from the disease have high levels of antibodies to the VSA while these levels are low and do not recognize most IEs at the onset or during infection in symptomatic individuals (Bull *et al.*, 1998; Ofori *et al.*, 2002; Hviid, 2005; Yone, Kremsner and Luty, 2005). There are several VSA expressed by *P. falciparum*, however the most well characterized is *P. falciparum* Erythrocyte Membrane Protein One (PfEMP1) encoded by *var* genes (Hviid and Jensen, 2015). While these immune targets may be correlates of exposure, VSA such as PfEMP1 have been implicated in cytoadhesive properties of IEs contributing to the pathology of the disease (reviewed in Hviid 2010; Bull and Abdi 2016).

1.2 Severe malaria

An estimated 70% of malaria deaths occur in children below 5 years who suffer from severe forms of the disease (WHO, 2015). In high transmission areas, it is estimated that 10-20% of all children will suffer from severe malaria before the onset of partial immunity (Gonçalves *et al.*, 2014). Why some children but not others develop severe malaria may be due to an interplay of host and parasite factors which will be discussed below.

Children in endemic areas are generally thought to be protected from malaria in the first 6 months of life (Dobbs and Dent, 2016). The transplacental transfer of maternal malaria antibodies have been suggested to passively

protect infants in the early months of life (Chizzolini *et al.*, 1989; Sehgal, Siddiqui and Alpers, 1989; Desowitz, Elm and Alpers, 1993), however, this association of protection with maternal antibodies is contentious. While antibodies to antigens such as CDRα1-containing PfEMP1 and MSP1 have been associated with protection, others such CSP, AMA1 showed an increased risk of disease or no association (Dobbs and Dent, 2016). The growth of parasites *in vitro*, has been shown to be inhibited by antibodies (IgA) and lactoferrin in breast milk and suggested to protect most of the children (Kassim *et al.*, 2000). However, IgG1 and IgG3 antimalarial antibodies are the antibody isotypes shown to be protective against severe malaria (Cohen, McGregor and Carrington, 1961; Targett, 1970; Garraud, Mahanty and Perraut, 2003; Duah *et al.*, 2010). These antibodies target antigens expressed by merozoites (such as AMA-1 and MSP1) and mature stage parasites (including PfEMP1) inhibiting invasion and cytoadherence of IEs by antibody mediated phagocytosis (Marsh *et al.*, 1989; Groux *et al.*, 1990; Chan *et al.*, 2012), antibody-dependent cellular inhibition (Bouharoun-Tayoun *et al.*, 1990, 1995; Tebo, Kremsner and Luty, 2001), or formation of immune complexes (Mibei *et al.*, 2008) which can also activate complement and other inflammatory mediators.

The risk of severe malaria in infants has been reported to be higher in children from multigravidas who had placental malaria than those from primigravidas (Mutabingwa *et al.*, 2005; Malhotra *et al.*, 2009; Gonçalves *et al.*, 2014). Malaria in multigravadas are uncommon in endemic areas, therefore, while this may correlate with the relatively few numbers of children who develop severe malaria, some of these studies relate parasitaemia to severity of disease and it is also unclear how factors such as parity, time of placental malaria and preventive treatment of placental malaria, have been adjusted for in these studies (Hviid, 2009; Dobbs and Dent, 2016).

Development of the immune system takes place within the first 2 years of life, however, the time taken for maturation differs between individuals (Holt and Jones, 2000; Jaspan *et al.*, 2006). As it has been described for allergies and autoimmunity (Holt and Jones, 2000), differences in development may affect control of immune responses to malaria infection in children in endemic areas

such that children whose immune system take a longer time to mature may have limited ability to raise or control immune responses to infection.

The level of fitness, co-infections, nutritional status of the children, use of bed nets which has been associated with lower risk of severe disease (Gonçalves *et al.*, 2014) and treatment at the time of infection may also contribute to differences in the outcome of infections.

Host polymorphisms may also account for differences in susceptibility to severe malaria. Blood group polymorphisms such as blood group O (Rowe *et al.*, 2007), sickle cell (HbS) (Aidoo *et al.*, 2002) , α+ thalassemia (Wambua *et al.*, 2006; Opi *et al.*, 2014), glucose-6-phosphate dehydrogenase deficiency (Ruwende *et al.*, 1995), are associated with protection from severe disease. These polymorphisms are common in Africa and may therefore protect majority of the children while the minority that lack these polymorphisms may be susceptible to severe disease.

Parasite specific factors such parasite multiplication rates (PMR), invasion pathways and repertoire of VSA could also influence the outcome of infection. High PMR were associated with severe malaria in adults (Chotivanich *et al.*, 2000) while no such association was observed in African children who had severe malaria compared to those with uncomplicated malaria (Deans *et al.*, 2006). These studies, which were based on *in vitro* PMR assays, are, however, limited in their ability to account for effects of multiple isolates during infection and effect of previous infections on PMR of the isolates.

The ability of parasites to invade erythrocytes by the sialic acid-dependent or sialic acid-independent pathways (Bowyer *et al.*, 2015; Mensah-Brown *et al.*, 2015) could also account for differences in outcome of infection, however, studies that examined the different pathways in clinical isolates reported no differences in invasion phenotypes between isolates from uncomplicated malaria and those from severe malaria (Deans *et al.*, 2007; Gomez-Escobar *et al.*, 2010).

The expression of specific types of PfEMP1 (group A or B/A) has been associated with severe malaria (Jensen *et al.*, 2004; Bull *et al.*, 2005; Kyriacou *et al.*, 2006; Lavstsen *et al.*, 2012). PfEMP1 is encoded by a repertoire of *var* genes;

it is possible that a combination of the host factors such as previous malaria infections (most severe malaria cases in children occur after at least two mild infections (Gonçalves *et al.*, 2014)) and co-infections with other diseases, could influence the expression of these group A or B/A PfEMP1 types that may enable the IEs to sequester in diverse organs and tissues to cause severe disease.

1.3 Cerebral malaria

Cerebral malaria, a major cause of malaria deaths, is defined as unarousable coma with Blantyre coma score ≤2 with *P. falciparum* detected in peripheral blood and no other evident cause of coma (WHO, 2014). Clinical presentation includes coma with respiratory distress, hypoxia, metabolic acidosis and seizures. Parasite killing antimalarial drugs are the main form of treatment for cerebral malaria, but about 15-20% cerebral malaria cases result in death and most survivors end up with neurological disorders (Idro *et al.*, 2010; John *et al.*, 2010; Miller *et al.*, 2013).

Pathological features of the disease include the excessive release or expression of proinflammatory cytokines IL-1β, IL-6 and TNF-α (Kern *et al.*, 1989; Kwiatkowski *et al.*, 1990; Molyneux *et al.*, 1993; Armah *et al.*, 2005). These cytokines may contribute to the disease through the activation of endothelial cells, increase in permeability of the blood brain barrier, upregulation of parasite adhesion receptors such as intercellular adhesion molecule 1 (ICAM-1) and increase sequestration in the brain microvasculature (Hunt and Grau, 2003).

Ring haemorrhages and localised coagulopathy characterized by fibrin deposits in the brain microvasculature have also been observed in brain sections from children who died from cerebral malaria (Seydel *et al.*, 2006; Moxon *et al.*, 2013; Milner *et al.*, 2014). IE binding to the endothelium may induce loss in Endothelial Cell Protein C Receptor (EPCR) and thrombomodulin (Moxon *et al.*, 2013) resulting in a procoagulant state.

Sequestration of mature IEs in the microvasculature of the brain is one of the key features of cerebral malaria pathology. Although there is sequestration in various organs and tissues in uncomplicated malaria cases, significantly high levels of sequestration of IEs has been observed in cerebral malaria cases, with most of the sequestration occurring in the brain (MacPherson *et al.*, 1985; Taylor *et al.*, 2004; Milner *et al.*, 2014). Children who were diagnosed with cerebral malaria but had little sequestration were found to have died of other non-malaria causes (Taylor *et al.*, 2004). Malaria retinopathy which is an indirect measure of sequestration in the brain that causes changes in colour of the ocular vessels, provides a better prognostic indicator of cerebral malaria than the current WHO diagnostic guidelines (Taylor *et al.*, 2004; Barrera *et al.*, 2015; Taylor and Molyneux, 2015). These data suggest that sequestration is a strong predictor or cause of fatal outcome of the disease, and perhaps it is so because of the associated dysregulation of the brain endothelium, breakdown of the blood brain barrier, increase in inflammation and coagulation (reviewed in Milner *et al.*, 2008) and impairment of tissue perfusion through the mechanical obstruction of the blood flow (Dondorp, Pongponratn and White, 2004; Dondorp *et al.*, 2008).

Comparisons of protein expressed by cerebral malaria isolates and uncomplicated or asymptomatic isolates from children in Benin have shown differences in the levels of proteins expressed. VSA such as PfEMP1 and RIFINS (encoded by repetitive interspersed family), and proteins involved in the trafficking and export of VSA to the surface of the IEs such as the Mature Parasite-infected Erythrocyte Surface Antigen (MESA) were relatively over expressed in the cerebral malaria isolates (Bertin *et al.*, 2016). While proteomic analysis (by mass spectrometry) overcomes the assumption in transcriptome analyses that all genes transcribed are expressed, the inability to identify peptides that are not in the mass spectrometry database is a limitation to such studies.

Parasites that transcribe a unique set of *var* genes that encode group A or B/A PfEMP1 (Kraemer and Smith, 2003; Lavstsen *et al.*, 2003) are associated with severe malaria including cerebral malaria (Jensen et al. 2004; Kyriacou et

al. 2006; Almelli et al. 2014a). A subset of the group A and B/A *var* genes that encode 'Domain cassette (DC) 8 and 13' type of PfEMP1 (described in section 1.4.1) have been associated with cerebral malaria in children (Bertin *et al.*, 2013; Almelli *et al.*, 2014b). These DC8 and DC13 variants were, however, identified using a set of 42 primers of which DC8 primers formed about 10%, the highest for a single DC, (Lavstsen *et al.*, 2012) and the set did not include all DC variants (Rask *et al.*, 2010). It is possible that these primers may be biased towards DC8 variants, and other DC variants transcribed by the clinical isolates that are not in the primer set would not be identified.

The DC8 and DC13 variants that have been associated with severe malaria were also transcribed by IEs that bind to human brain endothelial cells (HBEC), and antibodies from individuals recovering from cerebral malaria recognised these variants while antibodies at the time of infection did not (Avril *et al.*, 2012; Claessens *et al.*, 2012). IEs that express these variants have been reported to bind to EPCR, contributing to increase in coagulation and inflammation (Turner *et al.*, 2013). However, these studies have been largely based on, three laboratory adapted parasite lines and recombinant protein interactions.

1.4 Cytoadhesion of *P. falciparum* infected erythrocytes to host cells

Three cytoadhesion phenotypes have been described for the sequestration of *P. falciparum* infected erythrocytes (IEs) in the host microvasculature. Binding of IEs to uninfected erythrocytes to form rosettes, platelet mediated clumping of IEs and binding of IEs to endothelial cells (Rowe *et al.*, 2009) (Figure 1.2). IEs may exhibit any one of the above binding phenotypes (Claessens *et al.*, 2012), however, IEs that have dual binding phenotypes have also described (Adams *et al.*, 2014). The parasite ligand implicated in the binding to host cells, PfEMP1, and other VSA and the host receptors are discussed in the subsequent sections.

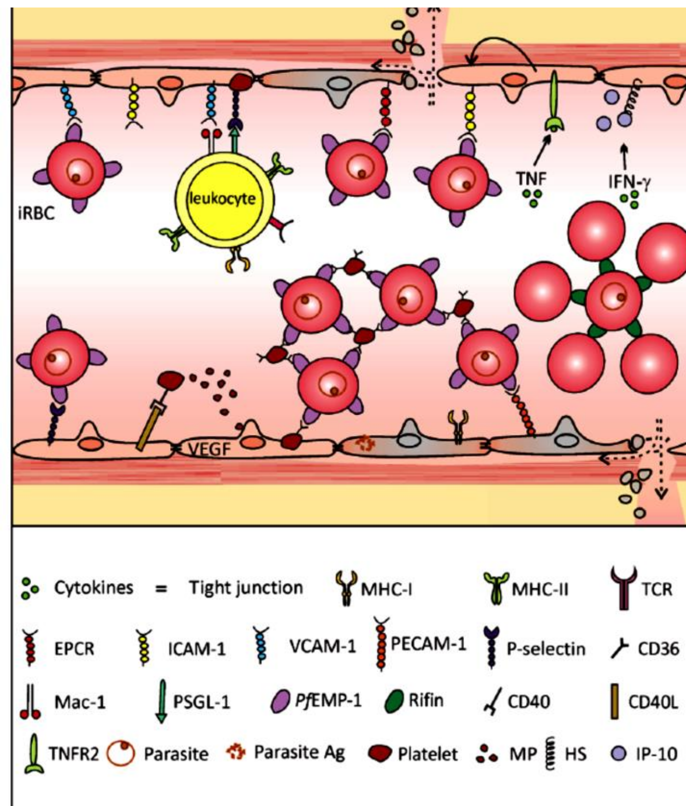


Figure 1.2. An illustration of sequestration of IEs in a blood vessel. Formation of rosettes, adhesion to receptors on endothelial cells, platelet mediated clumping, vascular leakage and endothelial cell activation are shown on the diagram. The diagram was modified from a figure taken from Deroost *et al.* (Deroost *et al.*, 2016).

1.4.1 PfEMP1 variant surface antigens

The parasite molecules thought to mediate IEs adhesion interactions are members of a large family of proteins transported to the surface of IEs, called PfEMP1. Each parasite genome has approximately 60 *var* genes encoding 60 variants of PfEMP1 (Baruch *et al.*, 1995; Smith *et al.*, 1995). The mature parasite expresses one of these different proteins at a time and can switch to any of them (hence a different phenotype) at each asexual cycle. Genetically distinct *P. falciparum* isolates have different *var* genes repertoires with very little overlap.

Var gene transcription is highest at the ring stage (Kyes, Pinches, and Newbold 2000), although *var* transcripts at all the erythrocytic stages have been

reported (Dahlbäck *et al.*, 2007). Expression of *var* genes is mutually exclusive, with one *var* gene being activated and expressed at a time, while the others are in a transcriptionally silent state (Dzikowski, Frank and Deitsch, 2006). *Var* gene antigenic switching rates of up to 3% per generation have been reported for cultured parasites, and these were independent of promotor or chromosomal location of the *var* genes. (Horrocks *et al.*, 2004; Fastman *et al.*, 2012).

Var genes are made up of two exons; the hypervariable exon 1 that encodes the extracellular region involved in adhesion interactions and the transmembrane, and the conserved exon 2, which encodes the acid terminal segment (ATS). *Var* genes (and hence PfEMP1) are classified into groups A, B, C and E based on their upstream sequence, chromosomal location and direction of transcription. Group A *var* genes are sub-telomeric and transcribed towards the telomere; group B *var* genes are also sub-telomeric but transcribed towards the centromere, while group C *var* genes are located in the central regions of the chromosome (Gardner *et al.*, 2002). The extracellular region of the PfEMP1 is made up of the N-terminal segment (NTS) and Duffy binding-like domains (DBL), cysteine rich interdomain regions (CIDR) (Figure 1.3). DBL domains can be classified into six types (α, β, γ, δ, ε and ζ) and the CIDR into four groups (α, β, δ and γ) (Smith *et al.*, 2000a; Rask *et al.*, 2010). Tandem domains of DBL-CIDR can also be classified as domain cassettes (DC) (Rask *et al.*, 2010). The domain cassettes may be functionally conserved.

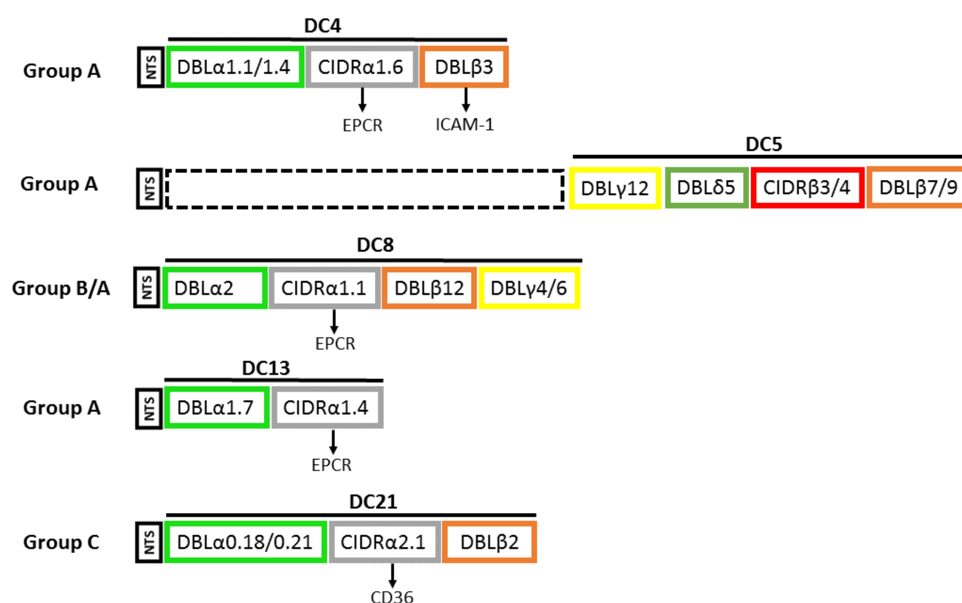


Figure 1.3. Domain architecture of DC4, DC5, DC8 and DC13 PfEMP1 showing some of the binding domains and their respective domain binding receptors. The structure of the domain cassettes were obtained from (Rask *et al.*, 2010; Bengtsson *et al.*, 2013) and the domain mapped receptors from (Bengtsson *et al.*, 2013; Smith *et al.*, 2013; Turner *et al.*, 2013; Lau *et al.*, 2015).

Binding of PfEMP1 domains to various host receptors have been described (reviewed in Kraemer and Smith, 2006; Smith *et al.*, 2013; Hviid and Jensen, 2015; Bull and Abdi, 2016). These include binding of the DBLβ domains of group A and B PfEMP1 to ICAM-1 (Smith *et al.*, 2000b; Bengtsson *et al.*, 2013; Gullingsrud *et al.*, 2013), CD36 binding of CIDRα2-6 domains of group B and C PfEMP1 (Baruch *et al.*, 1997; Robinson, Welch and Smith, 2003) and binding of the CIDRα 1.1 and 1.4-1.8 of group A and B/A PfEMP1 to EPCR (Turner *et al.*, 2013; Lau *et al.*, 2015) shown in Figure 1.3. These domains could serve as targets for the development of therapies against sequestration in severe malaria.

1.4.2 Other variant surface antigens

P. falciparum genome consists of other multigene families; repetitive interspersed family (*rif*), sub-telomeric variable open reading frame (*stevor*) and *P. falciparum* Maurer's clefts 2 transmembrane (*pfmc-2tm*) which encode RIFINS, STEVORS and PFMC-2TM respectively (Kyes *et al.*, 1999; Lavazec, Sanyal and Templeton, 2007; Joannin *et al.*, 2008). These proteins are expressed on the surface of IEs (Bachmann *et al.*, 2015). The role of RIFINS, STEVORS and PFMC-2TM in malaria is unclear due to the relatively few studies available (Abdel-Latif *et al.*, 2003; Niang, Xue and Preiser, 2009; Chan, Fowkes and Beeson, 2014; Niang *et al.*, 2014). RIFINS have been suggested to be involved in severe malaria due to its implication in formation of rosettes (Goel *et al.*, 2015), however, conflicting data have been reported on associations of rosette formation to severe disease (reviewed in Rowe *et al.*, 2009; Wang and Hviid, 2015). The role of these VSA in cytoadhesion and malaria needs to be explored further.

1.4.3 Receptors for cytoadhesion

IEs have been shown bind to various host receptors and attempts have been made to relate binding to specific receptors with disease. To date, with the exception of IE binding to chondroitin sulfate A (CSA) on syncytiotrophoblast in the placenta, which have been associated with malaria in pregnancy (Fried and Duffy, 2015), various association of receptors to severity or type of disease have been inconclusive (Rowe *et al.*, 2009; Craig, Khairul and Patil, 2012). The following would look at some of the widely studied receptors on host cells that IEs bind to.

1.4.3.1 CD36

IE binding to CD36 is probably the most studied IE-host receptor interaction. CD36 is an 85 kDa glycoprotein expressed on many types of cells including platelets, monocytes and endothelial cells. Isolates from all types of clinical presentation of malaria bind to CD36 and conflicting data on differences in binding between isolates from different types of malaria have been reported, however, most studies showed increase binding to C36 by uncomplicated malaria isolates (Ockenhouse *et al.*, 1989, 1991; Cojean *et al.*, 2008; Almelli *et al.*, 2014b; Cabrera, Neculai and Kain, 2014).

The PfEMP1 domain that binds to CD36 is the CIDRα2-6 of group B and C PfEMP1 (Baruch *et al.*, 1997; Miller *et al.*, 2002; Robinson, Welch and Smith, 2003; Janes *et al.*, 2011) and parasites from uncomplicated and asymptomatic malaria cases have been shown to predominantly transcribe group B and C *var* genes that encode the CD36 binding variants (Jensen *et al.*, 2004; Kyriacou *et al.*, 2006; Lavstsen *et al.*, 2012). IEs that bind to HBEC do not to bind to CD36 (Avril *et al.*, 2012; Claessens *et al.*, 2012) and CD36 is very sparsely or not expressed on HBEC (Turner *et al.*, 1994). A study of CD36 polymorphisms in African countries also reported no associations with severe malaria (Fry *et al.*, 2009).

These data suggest that IE binding to CD36 could explain the sequestration observed in tissues of individuals with uncomplicated malaria but not the brain-specific sequestration seen in cerebral malaria (Milner *et al.*, 2014).

1.4.3.2 ICAM-1

IEs bind to ICAM-1 expressed on endothelial cells (Berendt *et al.*, 1989), and activation of endothelial cells by cytokines, which results in upregulation of ICAM-1 on the surface of the cells (Wassmer *et al.*, 2011) may promote further binding and hence sequestration of IEs. Data on associations of ICAM-1 binding to severity of malaria have been conflicting (Berendt *et al.*, 1989; Ockenhouse *et al.*, 1991; A. Heddini *et al.*, 2001; Cojean *et al.*, 2008; Almelli *et al.*, 2014b).

IEs that bind to ICAM-1 express different groups of PfEMP1 containing the DBLβ binding domain that are encoded by groups A, B and C *var* genes (Oleinikov *et al.*, 2009; Janes *et al.*, 2011; Bengtsson *et al.*, 2013). Although HBEC-binding IEs had been previously reported not to bind to ICAM-1, (Avril *et al.*, 2012, 2013; Claessens *et al.*, 2012; Turner *et al.*, 2013) a recent study, using ICAM-1-transfected CHO cells, reports that the HBEC-binding IEs also bind to ICAM-1 by the DBLβ domain (Avril *et al.*, 2016). It appears binding to ICAM-1 may be involve in sequestration in different types of malaria but its specific role in severe malaria remains inconclusive.

1.4.3.3 EPCR

EPCR is a transmembrane protein expressed on endothelial cells that was recently found to be a receptor for IE binding to HBEC. Binding of severe malaria isolates to HBEC (compared to uncomplicated malaria isolates) has been shown to be mediated by EPCR (Turner *et al.*, 2013). IEs that bind to HBEC predominantly express DC8 and DC13 PfEMP1 (Avril *et al.*, 2012, 2013; Claessens *et al.*, 2012), which are associated with severe malaria in children and adults (Lavstsen *et al.*, 2012; Bertin *et al.*, 2013; Almelli *et al.*, 2014b; Bernabeu *et al.*, 2016). The CIDRα1 of DC8 and DC13 -expressing parasites that bind to HBEC and DC8 CIDRα1 of isolates from adult severe malaria cases bind to EPCR (Turner *et al.*, 2013; Lau *et al.*, 2015; Bernabeu *et al.*, 2016), and children in malaria endemic regions have also been reported to acquire antibodies to CIDRα1 domains that bind to EPCR early in life (Turner *et al.*, 2015).

EPCR forms a complex with thrombin to activate Protein C through the binding of Protein C to EPCR. Interactions of activated Protein C (APC) with Protease-activated receptor 1 (PAR1) is associated with anti-coagulative, anti-inflammatory and other cytoprotective effects (reviewed in Rao, Esmon and Pendurthi, 2014). Binding of CIDRα1 to EPCR has been shown to inhibit the activation of Protein C (Turner *et al.*, 2013; Lau *et al.*, 2015; Bernabeu *et al.*,

2016), and hence its associated cytoprotective effects contributing to the pathology of severe malaria.

Even though there has been huge advancement in identifying the EPCR and PfEMP1 binding domain and the binding sites (Turner *et al.*, 2013; Lau *et al.*, 2015), few published studies on IE binding to EPCR exist, therefore, more studies involving both laboratory and field isolates are required to determine how EPCR binding contributes to severe disease.

1.5 Aims of the thesis

This thesis investigates the effect of assorted environmental factors on the IE-HBEC binding interaction and examines the *var* genes expressed by IEs that bind to HBEC, the PfEMP1 binding domain and host receptors that could be mediating IE binding to HBEC.

1.5.1 Chapter 3

The hypothesis that environmental conditions such as pH, temperature, parasitaemia, flow conditions and serum would influence binding of IEs to HBEC is investigated. Optimal conditions for binding to HBEC are determined and any correlations of binding properties to cerebral malaria conditions are noted.

1.5.2 Chapter 4

The hypothesis that IEs that bind to HBEC predominantly express DC8 and DC13 PfEMP1 is tested using three Kenyan isolates recently adapted to culture. The DC8 and DC13 NTS.DBLα, CIDR1α and DBLβ domain recombinant proteins and antibodies, are also investigated in HBEC binding assays to try to determine which domain mediates binding to HBEC.

1.5.3 Chapter 5

The hypothesis that EPCR is the receptor for IE binding to HBEC is investigated. Binding of HBEC-binding IEs to other receptors (CD36, ICAM-1, platelet endothelial cell adhesion molecule 1 (PECAM-1), vascular cell adhesion protein 1 (VCAM-1), hyaluronan binding protein 1 (HABP-1), neural cell adhesion molecule 1 (NCAM-1), hyaluronic acid and CSA) that have been implicated in sequestration is also assessed.

2 Chapter 2: Materials and Methods

This chapter describes the materials and general methods for cell culturing, binding assays, fluorescent staining of IEs, selection of parasites for binding to brain endothelial cells and statistical analysis. Specific materials and methods have been included in subsequent chapters.

2.1 *P. falciparum* strains

Three Kenyan isolates, SAO75, 8211 and 9197 recently adapted to laboratory culture in 2009 (the sources of the 8211 and 9197 are described in Table 2.1; SAO75 was a gift from Jose Stoute) (Kifude 2013, PhD) and two well-established laboratory parasite strains; HB3, isolated from Honduras in 1980 (Bhasin and Trager, 1984) and IT4, isolated from Brazil in 1979 (Udeinya *et al.*, 1983) and possibly a product of cross contamination with the FCR3 strain (Robson *et al.*, 1992; Trager, 1993) were used.

Table 2.1 Details of the source of the Kenyan isolates

Sample	Disease	Clinical features	Age (months)
8211	Cerebral malaria	Impaired consciousness BCS = 2 Hb = 10 g/dl	34
9197	Severe malaria	Impaired consciousness BCS = 4 Hb = 7.1 g/dl	27

BCS – Blantyre coma score Hb – Haemoglobin

HB3 and IT4 had been previously selected for binding to HBEC-5i (Claessens and Rowe, 2012). The HBEC-binding HB3 parasite line predominantly expressed HB3var03 and is referred to as HB3var03 IEs while the HBEC-binding IT4 parasite line predominantly expressed IT4var07 or

IT4var19 (Claessens *et al.*, 2012). The IT4 parasite line was sorted by flow cytometry with IT4var07 or IT4var19 antibodies to generate two parasite lines referred to as IT4var07 IEs and IT4var19 IEs (done by Dr. Gabriella Lindergard). These three parasite lines are well characterized; surface staining of the IEs with antibodies to various domains of each of the PfEMP1 variants have been confirmed to validate parasite expression of those PfEMP1 variants (Rowe *et al*., unpublished data).

For the experiments done in this study, the percentage of IEs in the cultures expressing the PfEMP1 variants predominantly transcribed for HBEC binding (Claessens *et al.*, 2012) is shown in Table 2.2.

Table 2.2 PfEMP1 Expression of IEs in cultures used for experiments

Culture	Percentage of IEs expressing the PfEMP1 of interest
HB3var03 IEs	50 – 80 (HB3var03 – DC13 PfEMP1)
IT4var07 IEs	30 – 50 (IT4var07 – DC13 PfEMP1)
IT4var19 IEs	50 – 80 (IT4var19 – DC8 PfEMP1)

2.1.1 *P. falciparum* Culture

All work involving *P. falciparum* was done in a biosafety level 3 laboratory. Culturing was done in a Class II biological safety cabinet under sterile conditions. All materials used for culturing were sterile and the solutions were warmed up to 37°C for use.

2.1.2 General *P. falciparum* culturing

Parasites were cultured with O+ erythrocytes (Scottish National Blood Transfusion Service) in Roswell Park Memorial Institute (RPMI) 1640 medium (Lonza, BE12-167F) supplemented with 20 mM glucose (Scientific Laboratory Supplies, CHE1806), 25 mM HEPES (Lonza, 17-737F), 2 mM L-glutamine (Thermo Fisher Scientific, 25030), 25 μg/ml gentamicin sulfate (Lonza, 17-

518Z) (formed ‘incomplete RPMI medium’ for washes) and, 0.25% AlbuMAX® II Lipid-Rich BSA (Thermo Fisher Scientific, 11021), 5% pooled human serum (Scottish National Blood Transfusion Service), which formed the complete RPMI medium. Both incomplete and complete RPMI medium were adjusted with 1 M NaOH (Fisher Scientific, 10675692) to pH 7.2 - 7.3 for use. The cultures were incubated in a mixture of 1% O_2, 3% CO_2 and 96% N gas (BOC Gases) at 37^{0}C. Smears were made from approximately 250 µl of the culture daily (or after two days in some cases) and stained with Giemsa (TCS Biosciences, HS295) to determine the parasitaemia using light microscopy (described in detail below). The parasites were regularly diluted with O+ erythrocytes at the trophozoite stage to allow for reinvasion and to obtain the desired parasitaemia.

2.1.3 Washing of erythrocytes for culturing

Whole blood from donors was obtained weekly from the Scottish National Blood Transfusion Service. The white blood cells were removed by passing the blood through a leucodepletion filter attached to the blood pack. The recovered erythrocytes were then centrifuged at 2400g for 15 minutes, the supernatant discarded and washed twice at 20-30% haematocrit in incomplete medium at 2400g for 15 minutes and 10 minutes for the last wash. The erythrocytes were made up to 50% haematocrit for use and stored at 4°C for a maximum of 1 week.

2.1.4 Determination of parasitaemia by Giemsa staining

Approximately 250 µl of the parasite culture was centrifuged at 8500g for 7 seconds. The pellet of cells was reconstituted in approximately 30 µl of the supernatant to 30-50% haematocrit and about 10 µl smeared at an angle of about 45° on a clean glass slide. The smear was air dried, fixed with absolute methanol and air-dried. The slide was then flooded with filtered 10% v/v Giemsa in Giemsa buffer made up of PBS pH 7.2 (Merck, 109468), and left to stain for at least 15 minutes. The stain was gently washed off with water and

air-dried. The smear was viewed under oil with the 100X objective on a light microscope (Leica Microsystems, Leica DM1000). The number of IEs and uninfected erythrocytes were counted per field until a minimum total of 500 cells had been counted in at least 3 different fields.

2.1.5 Purification of IEs with knobs by gelatin flotation

Some parasites in long term culture tend to lose knobs expressed on the surface of the IEs (Kilejian *et al.*, 1986). To maintain the expression of knobs in culture, the parasites were regularly (at least one a month) purified by gelatin flotation described below. This purification separates mature pigmented trophozoites and schizonts IEs with knobs from those without knobs (hereafter referred to as 'knobless' IEs), uninfected erythrocytes and IEs with rings, thereby also synchronizing the parasite culture (Jensen, 1978). Cultures with predominantly mature pigmented trophozoite IEs were centrifuged at 800g for 4 minutes and the pellet made up to 40-50% haematocrit with incomplete medium in a 15 ml falcon tube. An equal volume of Gelofusine, (4% succinylated gelatin/0.007% NaCl in ddH_20, B. Braun Medical AB) was added to the cell suspension, mixed and incubated upright at 37°C for 15 minutes. Two layers of cells suspension formed. The bottom layer consisted of rings and knobless IEs, rosettes and uninfected erythrocytes. The top layer, containing the mature knob positive IEs, was gently taken up with a blunt pastette into a 15 ml falcon tube and centrifuged at 800g for 4 minutes. The pellet was washed (i.e. the pellet made up to 1 or 2% haematocrit with incomplete RPMI medium, centrifuged at 800g for 4 minutes and the supernatant aspirated) and the wash repeated with complete RPMI medium. A smear of the cells in the complete RPMI medium was made to determine the parasitaemia and success of the purification. The parasitaemia usually ranged from approximately 45% to 90%. The purified trophozoites IE with knobs were used for experiments or put back into culture at 2% haematocrit after dilution with erythrocytes to approximately 2% or the desired parasitaemia.

2.1.6 Cryopreservation of IEs

Culture to be frozen was centrifuged at 800g for 4 minutes and glycerolyte (57% glycerol, 0.14 M sodium lactate, 4 mM potassium chloride, 25 mM sodium phosphate) pH 6.8, added to the cells as follows: Glycerolyte, 0.33 times the pack cell volume (pcv) of the pellet, was added to the cells drop-wise and slowly with agitation to ensure uniform mixing of the suspension and left to stand. After 5 minutes, more glycerolyte, 1.33 times the cell pcv was added drop-wise and slowly to the suspension with agitation. The suspension was then aliquoted into cryotubes, stored at -70^{0}C and later transferred to liquid nitrogen for longer storage.

2.1.7 Thawing of IEs

Frozen IEs were thawed at 37°C and transferred to a 50 ml falcon tube. To 1 ml of the thawed IEs suspension, 200 µl of 12% NaCl was added drop-wise and slowly such that the process took at least 2 minutes and with each drop, the tube was agitated to ensure uniform mixing of the contents. The suspension was left for 5 minutes and 10 ml of 1.8% NaCl was added slowly and drop-wise with agitation, with the first 3 ml taking at least 5 minutes. After which 10 ml of 0.9% NaCl/0.2% glucose was also added drop-wise and slowly with agitation as above. The suspension was centrifuged at 800g for 4 minutes and the resulting pellet of cells washed with 20 ml of incomplete RPM1. The cells were put in culture at 2% haematocrit in complete RPMI medium, and an aliquot taken to make a smear to determine the parasitaemia. The culture was gassed with a mixture of 1% O_2, 3% CO_2 and 96% N for 30 seconds and incubated at 37^{0}C. The volumes of thawing solutions were adjusted proportionally depending on the volume of the thawed vial.

2.2 Human Brain Endothelial Cell line (HBEC-5i)

HBEC-5i is an immortalized endothelial line derived from microvessels of cortical fragments from a human brain (Dorovini-Zis, Prameya and Bowman, 1991; Wassmer *et al.*, 2006). Expression of surface receptors characteristic of primary HBEC such as PECAM-1, ICAM-1 and uptake of Dil-acetylated-low-density-lipoprotein (DiI-Ac-LDL) has been confirmed (Claessens, 2010, PhD; Rowe *et al.*, unpublished data). All work involving HBEC-5i were done in a biosafety level 2 or 3 laboratory. Culturing was done in a Class II biological safety cabinet under sterile conditions. All materials used for culturing were sterile and the solutions were warmed to 37°C for use.

2.2.1 General HBEC-5i culturing

HBEC-5i cells were grown on 0.1% gelatin coated tissue flask. The tissue culture flask was coated with gelatin by adding 3 ml of 0.1% v/v gelatin (Sigma-Aldrich, G1393) in dH_20 (for a T25 cm^2 flask) and laying the flask on the side to be coated at 37°C for at least 30 minutes after which the gelatin was aspirated. The cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM)/ Nutrient Mixture F-12 medium (Sigma-Aldrich, D6421) supplemented with 2 mM L-glutamine (Thermo Fisher Scientific, 25030), 100 U/ml penicillin/0.1 mg/ml streptomycin (Gibco, 15140), (to form incomplete DMEM medium), 10% v/v heat-inactivated foetal bovine serum, HI-FBS (Thermo Fisher Scientific, 10500064), 1% v/v endothelial cell growth supplement (ScienCell, 1052) and pH adjusted to 7.3 – 7.4 using 1 M NaOH (to form complete DMEM medium) at 37°C with 5% CO_2/air (referred to as 5% CO_2). The medium was changed every 48 hours or if the medium became acidic due to nutrient depletion (colour change from orange-red to yellow), whichever came first.

2.2.2 Passage of cells

The cells were passaged at 80-100% confluency by washing off the complete DMEM medium with incomplete DMEM medium and trypsinization to lift cells. The cells were then diluted into a new gelatin coated flask. In detail, for a T75 cm^2 tissue culture flask, the complete medium was aspirated and 10 ml of incomplete DMEM medium was used to wash the cells twice by adding the medium, swirling it over the adherent cells, aspirating off the medium and repeating the wash. Two ml of 0.25% trypsin-EDTA (Thermo Fisher Scientific, 25200) was added to the cells and incubated at 37°C for 2 to 3 minutes until all the cells were detached and 9 ml of complete DMEM medium was added to inhibit the trypsin activity.

The suspension of cells was centrifuged at 800g for 4 minutes. The supernatant was aspirated and the cells diluted with complete DMEM medium to the desired concentration (determined by a haemocytometer count). The cell suspension was then seeded onto T75 cm^2 gelatin coated flask in a total of 10 ml complete medium and incubated at 37°C with 5% CO_2. At least 4 hours after seeding, when the cells had evenly adhered to the gelatin on the flask, the medium was replaced with 20 ml fresh complete medium to get rid of dead and or floating cells and incubated at 37°C with 5% CO_2. The volumes were adjusted proportionally depending on the confluency of cells required and volume of the flask or plate used.

2.2.3 Freezing of HBEC-5i

Cells at 80-100% confluency were lifted with trypsin-EDTA as described above, and after washing in complete DMEM medium, they were resuspended in Cell Freezing Medium (ScienCell, 0133) to dilutions of at least 10^6 cells /ml. The cell suspension was aliquoted into cryotubes (1 ml each) and stored at -70^0C at least overnight before being transferred to liquid nitrogen.

2.2.4 Thawing of HBEC-5i

Frozen cells (1 ml) were thawed at 37°C and 4 ml of complete medium added to it. The cells in medium were centrifuged at 800g for 4 minutes and the supernatant aspirated. The cells were resuspended in 10 ml complete DMEM medium, seeded on a gelatin-coated T25 cm^2 flask and incubated at 37°C with 5% CO_2. The medium was replaced with 10 ml fresh medium the next day.

2.3 Human Brain Microvascular Endothelial Cells (HBMEC)

HBMEC (ScienCell, 1000) were cultured as described in section 2.2 for HBEC-5i, with the following exception. Endothelial cell medium, ECM (ScienCell, 1001) supplemented with 10 units/ml penicillin/streptomycin solution (ScienCell, 0503) was used for incomplete ECM medium and for the complete ECM medium, 5% foetal bovine serum, FBS, (ScienCell, 0025) and 1% endothelial cell growth supplement (ScienCell, 1052) was added to the incomplete medium. The pH of both media was adjusted to 7.3-7.4 with 1 M NaOH.

2.4 Static binding assays with HBEC-5i

HBEC-5i were seeded onto 0.1% w/v gelatin in dH_20 coated wells in a 48-well plate, at least one day before the assay, to reach 80 to 100% confluency on the day of the assay, with at least 2 wells per test. For the assay, parasites at the pigmented trophozoite stage (from culture or gelatin purified) and HBEC-5i were washed twice with DMEM binding medium (DMEM/Nutrient Mixture F-12 Ham (Sigma-Aldrich, D8900), supplemented with 2 mM L-glutamine and 100 U/ml penicillin/0.1 mg/ml streptomycin, pH 7.3). Two hundred µl of IEs at 2% haematocrit in 0.1% BSA (Sigma-Aldrich, A0336)/DMEM binding medium were added per well. The plate was incubated for 75 minutes at 37°C in air with gentle resuspension of cells after 30 and 60 minutes of incubation. The cells were washed with DMEM binding by gently rocking the plate thrice in four

directions, clockwise and anticlockwise, before the medium was gently taken off and replaced with fresh medium. The wells were checked, between washes, under an inverted microscope (Olympus CK2) to ensure there were few or no unbound infected or uninfected erythrocytes remaining. The cells were then fixed with 2% v/v glutaraldehyde (Sigma-Aldrich, G5882) in PBS (Sigma-Aldrich, P4417) for at least 1 hour and stained with 5% v/v Giemsa in Giemsa buffer (PBS pH 7.2) for 10 minutes. IEs bound to at least 50 HBEC-5i in not less than 3 random fields were counted by light microscopy using the 40X objective on the inverted microscope.

2.5 HBEC-5i or HBMEC static binding inhibition assay

Binding assays were carried out as described above (section 2.4), with a pre-incubation step in which the IEs or endothelial cells were incubated with antibodies or recombinant proteins in 0.1% BSA/DMEM binding medium for 20 minutes. For assays with IEs pre-incubated with antibodies or recombinant proteins, 190 µl of the IE-antibody/protein suspension (2% haematocrit) was added to the HBEC-5i or HBMEC in each well, and for assays with HBEC-5i or HBMEC pre-incubated with antibodies or recombinant proteins, IEs were added to 190 µl of the diluted antibody or protein in the well to make up 2% haematocrit in each well. The assay was done as described above with an hour incubation. Binding was compared with control wells that contained IEs and HBEC-5i or HBMEC in 0.1% BSA/DMEM binding medium with appropriate isotype controls or in 0.1% BSA/DMEM binding medium only.

2.6 Binding of IEs to receptor recombinant proteins and glycosaminoglycans

Recombinant proteins and glycosaminoglycans were diluted to 50 µg/ml in PBS and 3 µl of the diluted recombinant protein was spotted on two untreated Petri dish (BD Falcon 351007), two spots per dish in a radial fashion at an equal

distance from the centre to ensure equal and maximum exposure of the IEs to the receptors, and incubated overnight at 4°C. The proteins were aspirated and the plate was blocked with 2 ml 3% BSA/PBS for 2 hours at 37°C. The 3% BSA/PBS was taken off and the dish was washed with 2 ml of DMEM binding medium. Pigmented trophozoites, straight from culture or gelatin purified, were washed twice with DMEM binding medium and made up to 2% haematocrit in 0.1% BSA/DMEM binding medium. Two ml of the IEs suspension was added to each dish and the dishes were incubated at 37°C in air for 1 hour, with resuspension by swirling every 12 minutes. The dish was washed gently by taking off the IE suspension and adding DMEM binding medium. The dish was gently swirled four directions, clockwise and anticlockwise and the medium was taken off and the wash repeated with regular checks under an inverted microscope till there were no more unbound cells. The adherent IEs were fixed with 2% glutaraldehyde in PBS for 1 hour, and stained with 5% Giemsa in Giemsa buffer (PBS pH 7.2) for 10 minutes. Bound IEs on 3 fields were counted for each spot with a light microscope using the 40X objective on the inverted microscope.

2.7 Fluorescence-activated cell sorting (FACS) of IEs expressing a specific PfEMP1

IEs expressing the PfEMP1 of interest, required for binding to HBEC-5i were regularly sorted from culture by FACS to enrich for a more homogeneous population of IEs expressing that PfEMP1 in culture. Mature pigmented trophozoite IEs were purified by magnetic-activated cell sorting (MACS) before FACS. The VarioMACS Separator (Miltenyi Biotec, 130-090-282), which uses a magnet to separate IEs in culture based on the paramagnetic properties of the hemozoin in mature stage parasites, was used in combination with CS Columns (Miltenyi Biotec, 130-041-305) according to the manufacturer's protocol.

Briefly, the parasite culture was centrifuged at 800g for 4 minutes, the supernatant was discarded and 20 ml of incomplete RPMI medium added to the

pellet and centrifuged again at 800g for 4 minutes. The supernatant was discarded and the cells were resuspended in 20 ml of incomplete RPMI medium. The column, a 20G needle and a 20 ml syringe were fitted onto a three-way tap. The column was fitted into the magnet and 30 ml of incomplete RPMI medium added to the syringe. Ten ml of incomplete RPMI medium in the syringe was forced up and down the column to get rid of any trapped air in the column and allowed to run through the tap at one drop per second. Another 10 ml of incomplete RPMI medium was also added directly to the column to run through the tap at the same rate.

The parasite suspension was added to column and run through the tap at 1 drop per second. The flow through was collected and run through the column a second time. The column was washed with 15 to 20 ml of incomplete RPMI medium till the flow through was clear. The column with the tap and syringe attached was removed from the magnet and the 20 ml incomplete RPMI medium in the syringe forced repeatedly up and down the column to elute the bound mature pigmented IEs.

The recovered IEs were pulled into the syringe and transferred to a 50 ml falcon tube. The elution was repeated with another 20 ml of incomplete RPMI medium in a syringe and the recovered IEs were added to the falcon tube containing the first eluent. Five hundred µl of the suspension was centrifuged at 8500g for 7 seconds, reconstituted to 30-50% in 20 µl of the supernatant and 10 µl used to make a smear to determine the parasitaemia and success of the MACS (usually 80-90% matured pigmented parasites). The purified IEs suspension was centrifuged at 800g for 4 minutes, the supernatant was aspirated and 10 ml of PBS added to the pellet to wash the IEs. The suspension was centrifuged at 800g for 4 minutes, the supernatant was aspirated and the IEs were made up to 3.5 ml in 1% BSA/PBS.

A 500 µl aliquot was set aside as a “no antibody” control. To the remainder of the IEs, rabbit polyclonal IgG to the PfEMP1 of interest (Claessens *et al.*, 2012) was added at a final concentration of 25 µg/ml and incubated at room temperature for 45 minutes with resuspension every 10 minutes to ensure equal chance of staining for all the cells. The suspension was centrifuged

at 800g for 2 minutes and the supernatant aspirated. The cells were washed by resuspension in 3 ml of PBS, centrifuged at 800g for 2 minutes and the supernatant aspirated. The wash was repeated twice. Five hundred µl and 3 ml of Alexa Fluor 488 goat anti-rabbit IgG (H+L) (Thermo Fisher Scientific, A-11008) at 1/1000 in 1% BSA/PBS were added to the 'no antibody control' and to IEs stained with the primary antibody respectively, and incubated at room temperature for 45 minutes. After which the cells were washed thrice in PBS and made back up to 500 µl ("no antibody" control) and 3 ml (stained IEs) with complete RPMI medium.

The IEs that stained positive for the PfEMP1 antibody were sorted with BD FACSAria I using the BD FACSDiva 6 software (BD Biosciences). An acquisition gate was applied to the forward scatter plot for the exclusion of debris from intact cells. From the acquisition of cells at approximately 20,000 events per second, IEs that stained positive for Alexa Fluor 488 (excitation at 488 nm and emission between 525-530 nm) were sorted at a system pressure through a 70 µm nozzle into 7 ml of complete RPMI media with 30% pooled human serum in 15 ml tubes. The sorted IEs were centrifuged for 4 minutes at 800g and washed with incomplete RPMI medium. The IEs were put back into culture in complete RPMI medium with fresh erythrocytes at 0.5% haematocrit, gassed for 30 seconds with a mixture of 1% O_2, 3% CO_2 and 96% N gas and incubated at 37°C.

2.8 Flow cytometry to determine PfEMP1 expression of IEs

A culture of mature pigmented trophozoites at 2% haematocrit was used for the flow cytometry. An aliquot of 1.5 ml of the culture was centrifuged at 8500g for 7 seconds and washed thrice in PBS. The cells were resuspended in 1.5 ml of PBS/1.25 µg/ml Hoechst (Thermo Fisher Scientific, H3570) and incubated at 37°C for 20 minutes. The suspension was centrifuged and the cells washed with 750 µl of PBS. The volume of cells was made up to 1.2 ml in PBS/1% BSA/1.25 µg/ml Hoechst. Tubes of cell suspension with a final volume of 100 µl each

were set up as follows, a no antibody control, 25 µg/ml of rabbit IgG control and 25 µg/ml rabbit polyclonal IgG to the PfEMP1. The samples were incubated on ice for 1 hour and resuspended every 10 minutes.

The cells in each tube were centrifuged at 8500g for 7 seconds, supernatant aspirated and the cells washed thrice with 750 µl of cold PBS. One hundred µl of secondary antibody, Alexa488 goat anti-rabbit IgG at 1/1000 in PBS/1% BSA/ 1.25 µg/ml Hoechst was added to the cells in each tube. The samples were incubated on ice in the dark for 1 hour and resuspended every 10 minutes. For each tube, the cells were centrifuged, supernatant aspirated and the cells washed thrice with cold PBS. The cells were resuspended in 200 µl of 0.5 % v/v formaldehyde (Thermo Fisher Scientific, 28906)/PBS and incubated on ice for 20 minutes. They were washed in 750 µl of cold PBS and washed again in 750 µl of 0.1% BSA/0.1% azide in PBS (FACS buffer). Five hundred µl of FACS buffer was added to the cells in the tubes and transferred to a FACS tube.

The tubes were protected from light and the flow cytometric analysis done with the BD LSR II flow cytometer with 355 and 488 nm lasers using the BD FACSDiva 6 software (BD Biosciences) immediately or the samples were stored at 4°C for a maximum of 24 hours until analysis could be done. For the analysis, an acquisition gate was applied to the forward scatter plot for the exclusion of debris from intact cells and 30,000 events were acquired. DAPI stained parasite nuclei was detected by excitation at 355 nm and emission between 450/40 nm, and Alexa Fluor 488 positive IEs were identified following excitation at 488 nm and emission between 525-530 nm. Data acquired was analysed using FlowJo software (Tree Star).

2.9 Immunofluorescence assay with PfEMP1 antibodies

About 500 µl of mature pigmented trophozoites in culture, was centrifuged at 8500g for 7 seconds and washed twice in 1 ml of 1% BSA/PBS. The cells were made up to 2% haematocrit in 1% BSA/PBS. Rabbit polyclonal IgG to the PfEMP1 or rabbit IgG (isotype control) in PBS was added to the cell suspension

to give an antibody concentration of 20 µg/ml in 50 µl. A “no antibody” control, which was made up of 50 µl of the cells suspension in 1% BSA/ PBS only, was included. The samples were incubated on ice for 1 hour and resuspended every 10 minutes. The cell suspensions were centrifuged at 8500g for 7 seconds, the supernatant aspirated and the cells washed in 750 µl of cold PBS by mixing the cells and centrifuging them at 8500g for 7 seconds. The supernatant was aspirated and the wash repeated twice, after which 50 µl of Alexa488 goat anti-rabbit IgG at 1/1000 in 1% BSA/ PBS/ 1 µg/ml 4,6-diamidino-2-phenylindole, DAPI, (Molekula, M70277046) was added to each test sample and incubated on ice for 1 hour in the dark with resuspension of the samples every 10 minutes.

The cell suspensions were centrifuged at 8500g for 7 seconds, the supernatant was aspirated and the cells washed thrice in cold PBS as described above. The cells were resuspended to 20-30% haematocrit in 1% BSA/ PBS and smears were made for each test sample. The smears were air-dried and a drop of 2.5 mg/ml 1,4-diazabicyclo[2.2.2]octane, DABCO (Sigma-Aldrich, 290734)/ glycerol in PBS was added to each smear and covered with coverslip. Nail varnish was used to seal the ends of the coverslip to prevent the smears from drying out. The smears were stored at 4°C at least overnight and viewed under the fluorescent microscope. The percentage of IEs with the characteristic punctate fluorescent of PfEMP1 in at least 100 IEs were counted in a minimum of 4 fields under 100X on a Leica DM LB2 microscope (Leica Microsystems).

2.10 Statistical analyses

Statistical analyses were done using GraphPad Prism. A parametric paired t test was used for comparisons between two samples. For experiments with multiple groups, a repeated measures one-way ANOVA was used to compare the means of three or more groups of biological replicates while an ordinary one-way ANOVA was used for experiments with two biological replicates.

Depending on the aim of the experiment, comparisons of the means to every other mean were done using a Tukey’s test, or a Dunnett’s test was used

for comparisons of the means to that of the control. For experiments, which the controls varied widely between biological replicates, the values obtained were log transformed to normalise the values before the statistical analyses were done. Statistical differences are shown by * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ and **** $P < 0.0001$).

2.11 List of Materials

Material	Supplier: order number
Cells	
HBMEC	ScienCell: 1000
One Shot TOP10F´ *E. coli*	Thermo Fisher Scientific: C3030
Recombinant Proteins and Glycosaminoglycans	
EPCR recombinant protein	Sino Biologicals: 13320-H02H
CD36	R&D systems: 1955-CD
CSA	Sigma-Aldrich: 27042
HABP-1	R&D systems: 5155-HB
Heparin	Sigma-Aldrich: H4784
Hyaluronic acid	Sigma-Aldrich: H7630
ICAM-1	R&D systems: 720-IC
NCAM-1	R&D systems, 2408-NC
PECAM-1	Sino Biologicals: 10148-H02H
VCAM-1	R&D systems, 862-VC
Antibodies	
Alexa Fluor 488 goat anti-rabbit IgG (H+L)	Thermo Fisher Scientific: A-11008
Alexa Fluor 488 goat anti-mouse IgG (H+L)	Thermo Fisher Scientific: A- A11029
Alexa Fluor 488 donkey anti-goat IgG (H+L)	Thermo Fisher Scientific: A-11055
Amplitaq Gold DNA Polymerase	Thermo Fisher Scientific: 4311816
EPCR monoclonal antibody	Hycult, HM2145
EPCR polyclonal antibody	R&D systems: AF2245
Goat IgG	R&D systems: AB-108-C
ICAM-1 monoclonal antibody	AbD Serotec: MCA1615XZ
Mouse IgG	Sigma-Aldrich: MOPC-31c
Rat IgG	Hycult, HI3001
Reagents	
Agarose	Thermo Fisher Scientific: 15510-027
0.25% AlbuMAX® II Lipid-Rich BSA	Thermo Fisher Scientific: 11021
BigDye	Thermo Fisher Scientific: 4337457
BSA	Sigma-Aldrich: A0336
Cell Freezing Medium	ScienCell: 0133

Material	**Supplier: order number**
Chloroform	Sigma-Aldrich: C2432
Control A siRNA	Santa Cruz: sc-37007
Control A siRNA- FITC	Santa Cruz: sc-36869
EPCR siRNA	Santa Cruz: sc-39932
DABCO	Sigma-Aldrich: 290734
DAPI	Molekula: M70277046
DNAse 1/DNAse buffer/EDTA	Thermo Fisher Scientific: 18068-015
DNA Ladder (PCR Ranger 100 bp)	Norgen: 11300
DNA Ladder (*FullRanger* 100 bp)	Norgen: 11800
DNA Ladder (Quick-Load 100 bp)	New England Biolabs: N0467S
DNA loading dye	Qiagen: 239901
DMEM/Nutrient Mixture F-12 Ham	Sigma-Aldrich: D6421
DMEM/Nutrient Mixture F-12 Ham without $NAHCO_3$	Sigma-Aldrich: D8900
Dynabeads M-450 Epoxy	Thermo Fisher Scientific: 14011
Endothelial cell medium (ECM)	ScienCell: 1001
Endothelial cell growth supplement	ScienCell: 1052
FBS	ScienCell: 0025
Formaldehyde	Thermo Fisher Scientific: 28906
Gelatin	Sigma-Aldrich: G1393
Gelofusine	B. Braun Medical AB
Gentamicin sulfate	Lonza: 17-518Z
Giemsa	Biosciences: HS295
Giemsa buffer	Merck: 109468
Glucose	Scientific Laboratory Supplies: CHE1806
L-Glutamine	Thermo Fisher Scientific: 25030
Glutaraldehyde	Sigma-Aldrich: G5882
HEPES	Lonza: 17-737F
HI-FBS	Thermo Fisher Scientific: 10500064
Hoechst	Thermo Fisher Scientific: H3570
LB-agar	Sigma-Aldrich: L2897

Material	Supplier: order number
LB-Broth	Sigma-Aldrich: L3022
Lidocaine hydrochloride	AppliChem: A2870
Lipofectamine RNAiMAX transfection reagent	Thermo Fisher Scientific, 13778030
NaOH	Fisher Scientific: 10675692
OPD	Sigma-Aldrich: P8787
Opti-MEM reduced serum medium	Thermo Fisher Scientific: 31985062
Penicillin/ streptomycin	Gibco: 15140
Penicillin/streptomycin solution (for ECM)	ScienCell: 0503
PBS	Sigma-Aldrich: P4417
QIAprep Spin Miniprep Kit	Qiagen: 27106
Qiaquick PCR purification kit	Qiagen: 28104
RPMI	Lonza: BE12-167F
SuperScript III First-Strand Synthesis System	Thermo Fisher Scientific: 18080-051
SuperScript III Reverse Transcriptase	Thermo Fisher Scientific: 18080-044
S.O.C medium	Thermo Fisher Scientific: 15544-034
TA Cloning Kit, Dual Promoter, with PCR II Vector	Thermo Fisher Scientific: K2070
T4 DNA ligase	Thermo Fisher Scientific: 15224-041
TBE Buffer	Thermo Fisher Scientific: 15581-044
TRIzol	Thermo Fisher Scientific: 15596026
Trypsin-EDTA	Thermo Fisher Scientific: 25200
Tween-20	Sigma-Aldrich: P9416

3 Chapter III: Adhesion properties of *P. falciparum* infected erythrocytes that bind to HBEC-5i

3.1 Abstract

Cerebral malaria is characterized by the sequestration of mature *P. falciparum* infected erythrocytes (IEs) in microvasculature of the brain. The IEs sequester via variant surface antigen, PfEMP1 encoded by *var* genes, expressed on the surface of the IEs. PfEMP1 of domain cassettes (DC) 8 and 13, have been identified as the parasite ligand responsible for the cytoadherence of IEs to brain endothelial cells. However, knowledge of the parasite and host environmental conditions that can affect cytoadhesion is lacking and there are no established conditions for the *in vitro* assay based on this parasite binding interaction with brain endothelial cells, a model for studies on cytoadherence in cerebral malaria. Using a human brain endothelial cell line, HBEC-5i, three parasite lines expressing DC8 or 13 PfEMP1 and a clinical isolate recently adapted to culture, the effect of gaseous conditions, pH, temperature, parasitaemia and serum on the cytoadherence of IEs to HBEC-5i was investigated. Binding of the IEs to HBEC-5i under flow was also evaluated. Cytoadhesion was found to be maximal at pH 7.3, parasitaemia-dependent and was not significantly affected by increase to febrile temperature or hypoxic conditions. Normal human serum reduced adhesion to HBEC-5i in all the four strains; however, it abolished cytoadhesion of the EPCR-binding variant IT4var19. The parasites, previously selected for HBEC-binding under static conditions, also bound to HBEC-5i under flow. This study not only informs about effects of the environment on adhesion, but also presents optimal conditions for the *in vitro* adhesion of IEs to brain endothelial cells.

3.2 Introduction

Cerebral malaria is a major cause of the 438,000 annual malaria deaths, affecting mostly children below 5 years infected with *P. falciparum* in sub-Saharan Africa (Jallow *et al.*, 2012; WHO, 2015). The major pathology implicated in cerebral malaria is the sequestration of mature IEs in microvasculature of the brain (Taylor *et al.*, 2004; Seydel *et al.*, 2006; John *et al.*, 2010; Hanson *et al.*, 2012). Autopsy-based studies show a strong correlation between sequestration of IEs in the microvasculature of the brain and cerebral malaria that is distinct from other forms of the disease (Ponsford *et al.*, 2012; Milner *et al.*, 2014).

Mature IEs bind to host endothelial cells to avoid splenic clearance and for optimal growth in the low oxygen tension environment resulting in the obstruction of the microvasculature (Dondorp, Pongponratn, and White 2004). The parasite variant surface protein implicated in this interaction is PfEMP1 encoded by *var* genes. Although other variant surface antigens such as RIFINS and STEVORS have also been implicated in cytoadherence, the most evidence has been shown for PfEMP1 (Smith *et al.*, 1995; Sherman, Eda and Winograd, 2003; Kyes, Kraemer and Smith, 2007; Claessens *et al.*, 2012).

PfEMP1 variants are classified into groups A-E based on their upstream sequence or into domain cassettes (DC) based on the arrangement of their domains. Parasites that express PfEMP1 of group A and B/A containing DC8 and DC13 variants have been associated with severe disease in children (Lavstsen *et al.*, 2012; Bertin *et al.*, 2013; Smith *et al.*, 2013; Almelli *et al.*, 2014b; Tembo *et al.*, 2014). These same PfEMP1 variants are predominantly transcribed by IEs that bind to HBEC (Avril *et al.*, 2012, 2013; Claessens *et al.*, 2012).

Also essential for cytoadhesion to endothelial cells are knobs on the surface of the IEs (Udeinya *et al.*, 1981; Langreth and Peterson, 1985; Crabb *et al.*, 1997; Claessens *et al.*, 2012). Knobs, made up of knob associated histidine-rich protein (KAHRP), are cup-like protrusions that have been shown to localise or tether PfEMP1 on the surface of the IEs for binding to endothelial cells (Baruch *et al.*, 1995; Ganguly *et al.*, 2015). The density of knobs on the IEs have

also been shown to be associated with the type of PfEMP1 variant expressed (Subramani *et al.*, 2015). Although IEs without knobs also appear to bind to host cells receptor proteins such as CD36 under static conditions (Biggs *et al.*, 1990; Tilly *et al.*, 2015), knobs seem to be essential for binding under flow (Crabb *et al.*, 1997).

A number of host endothelial cell surface proteins, including ICAM-1, have been suggested to be receptors involved in the cytoadherence in the brain (Xiao *et al.*, 1996; Rogerson *et al.*, 1999; Cserti-Gazdewich *et al.*, 2012; Almelli *et al.*, 2014b), though no strong evidence for this has been shown. The current leading candidate receptor is the endothelial protein C receptor, EPCR (Turner *et al.*, 2013), however, evidence described in chapter 5 and from other reports (Schuldt *et al.*, 2014; Nunes-Silva *et al.*, 2015) suggests that this may not be physiologically relevant or true for all HBEC-binding parasite lines.

Both genetic and environmental factors of the host and parasite may play a significant role in the outcome of cerebral malaria. To date, there have been relatively many studies on host and parasite genetic factors that could affect or predict the outcome of disease, however, environmental factors within the host and surrounding the parasite that may affect this unique cytoadherence of IEs to HBEC in cerebral malaria have not been extensively studied. Changes in host and parasite environment that have been associated with cerebral malaria include febrile temperature, acidosis, proinflammatory cytokines such as tumour necrosis factor α (TNFα), and IFNγ (interferon γ) (reviewed in Combes *et al.*, 2010). Relatively few studies have looked at these factors in relation to the cytoadherence of IEs to brain endothelial cells, and most of these studies were not based on the cytoadhesion of the DC8 and DC13 -expressing parasite lines, with the exception of two studies that looked at the effect of TNFα on adhesion using these parasites (Claessens *et al.*, 2012; Gillrie *et al.*, 2015).

Cerebral malaria affects the brain of infected individuals, which makes it difficult to study in living humans. Interactions between parasite and host cells have been studied in autopsies and murine models in order to understand the pathogenesis of cerebral malaria. While these studies are useful, it is difficult to obtain samples for autopsy studies, and murine models are not fully

representative of cerebral malaria in humans (Franke-Fayard *et al.*, 2010; White *et al.*, 2010; Craig *et al.*, 2012). Hence, the use of endothelial cells derived from human brain tissues and IEs *in vitro* may provide a better model for cytoadherence in cerebral malaria. Knowing the effect of environmental conditions on cytoadhesion would enable the establishment of optimal conditions for this *in vitro* model.

3.3 Hypothesis and specific aims

This chapter investigates the hypothesis that changes in environmental conditions would affect cytoadhesion of all HBEC-binding IEs and that optimal binding conditions would be similar for all HBEC-binding IEs.

Specific aims

1. To investigate the effect of the following environmental conditions; gaseous conditions, pH, temperature, parasitaemia and serum, on adhesion of IEs to HBEC-5i and hence determine the optimal binding conditions.
2. To evaluate the effect of gelatin purification for IEs with knobs on cytoadhesion to HBEC-5i
3. To determine whether IEs selected for binding to HBEC-5i under static conditions would also bind under flow conditions.

3.4 Methods

3.4.1 Medium for assays

Media used for the assays (unless stated otherwise) are described as follows.

i. $NaHCO_3$-free DMEM (DMEM binding medium) – DMEM/Nutrient Mixture F-12 Ham (1.56% w/v in dH_20) containing 15 mM HEPES without

$NaHCO_3$ (Sigma-Aldrich, D8900), supplemented with 2 mM L-glutamine and 100 U/ml penicillin/0.1 mg/ml streptomycin. The pH was adjusted to 7.3 with drops of 1 M NaOH.

ii. $NaHCO_3$-buffered DMEM (incomplete DMEM medium) – DMEM/Nutrient Mixture F-12 Ham containing 15 mM $NaHCO_3$ and 15 mM HEPES (Sigma-Aldrich D6421), supplemented with 2 mM L-glutamine, 100 U/ml penicillin/0.1 mg/ml streptomycin and pH made up to 7.3 with drops of 1 M NaOH.

3.4.2 Binding of IEs to HBEC-5i in different gaseous conditions

IEs from a culture of at least 5% pigmented trophozoites were gelatin purified and three 48 well plates, each with three wells of 80 to 100% confluent HBEC-5i were washed twice with $NaHCO_3$-buffered DMEM medium pH 7.3. The plates were each labelled as CO_2, parasite gas or air. The parasites (at least 30% parasitaemia) were resuspended to 2% haematocrit in the 0.1% BSA/$NaHCO_3$-buffered DMEM medium and added to the HBEC-5i; 200 µl per well. The 'CO_2' plate was incubated at 37°C in a 5% CO_2 incubator, the 'parasite gas' plate was put into a sealed gas chamber and gassed with a mixture of 1% O_2, 3% CO_2 and 96% N gas (hereafter referred to as 'parasite gas') for at least 2 minutes and together with the 'air' plate (incubated in air; 78.09% nitrogen, 20.95% oxygen, 0.93% argon, 0.039% CO_2 and other gases), both were incubated at 37°C in an incubator for 75 minutes with a gentle resuspension after every 30 minutes.

Unbound IEs were gently washed off with the $NaHCO_3$-buffered DMEM medium, by gently rocking the plate thrice in four directions, clockwise and anticlockwise, before the medium was gently taken off and replaced with fresh medium. The washes were repeated thrice and the plate was checked under an inverted microscope for any unbound IEs remaining. The washes were repeated with the plate checked between washes till there were few or no unbound cells remaining in the medium. The cells were fixed with 2% glutaraldehyde in PBS for 1 hour and stained with 5% v/v Giemsa in PBS pH 7.2 for 10 minutes. IEs

bound to at least 50 HBEC-5i (Figure 3.1) were counted by light microscopy at 400X magnification on the inverted microscope.

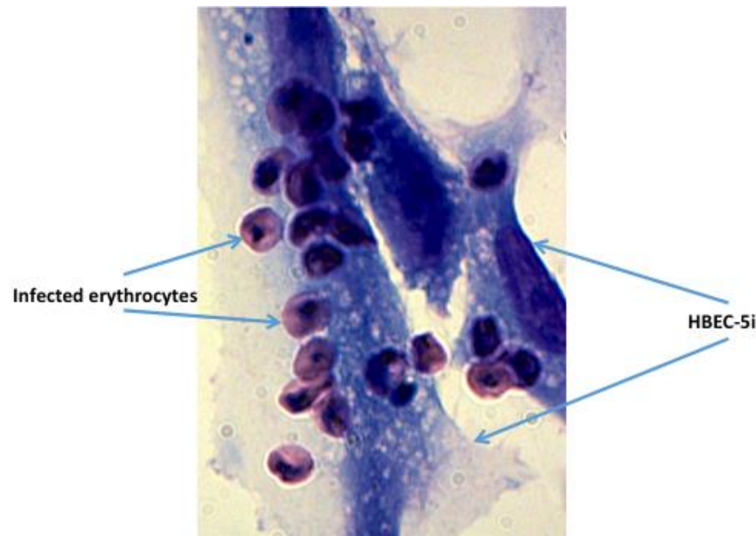


Figure 3.1. Adhesion of HB3var03 IEs to HBEC-5i. Gelatin purified trophozoites were co-incubated with HBEC-5i in 0.1% BSA/$NaHCO_3$-free DMEM medium pH 7.3 at 37°C for 75 minutes. Picture was taken at 1000X magnification.

3.4.3 HBEC-5i binding assay at varying pH

One hundred and fifty ml of $NaHCO_3$-free DMEM medium (pH not adjusted) was dispensed into each of 7 bottles. The pH of the medium in each bottle was adjusted to 6.8, 7.0, 7.2, 7.3, 7.4 or 7.6 with 1 M NaOH and filtered sterile. The bottles were each labelled with the corresponding pH and media warmed up to 37°C for use or stored at 4°C.

IEs from a predominantly matured pigmented trophozoite culture (parasitaemia and pcv of at least 5% and 500 µl respectively) were gelatin purified and washed with the $NaHCO_3$-free DMEM medium pH 7.3. The supernatant was discarded and the pellet resuspended to 2% haematocrit in the $NaHCO_3$-free DMEM medium pH 7.3. The parasite suspension, at not less than 30% parasitaemia, was divided into 6 tubes with at least 0.5 ml in each tube labelled with the different pHs (6.8, 7.0, 7.2, 7.3, 7.4 or 7.6). The tubes were

centrifuged at 800g for 4 minutes and the supernatant discarded. The pellets in the tubes were resuspended to the same volume (to make up the 2% haematocrit) with 0.1% BSA/$NaHCO_3$-free DMEM medium of a pH corresponding to the label on the tube.

The HBEC-5i in 12 wells of a 48 well plate at 80 – 100% confluency were washed twice with $NaHCO_3$-free DMEM medium pH 7.3 and the wells labelled; 2 wells per pH. Two hundred µl of IEs in the tubes were added to each well labelled with their corresponding pH and the plate was incubated at 37°C for 75 minutes in air. The plates were gently rocked to resuspend the IEs every 30 minutes. Unbound IEs were gently washed off with their respective pH medium, till there were few or no unbound cells in the medium. The cells were fixed, stained and IEs bound to at least 50 HBEC-5i counted as described in section 3.42.

For one of the experiments, tubes containing 20 ml of 0.1% BSA/$NaHCO_3$-free DMEM at the different pHs were also incubated at 37°C alongside the cells in air. The pH of the media in each tube was measured after 75 minutes of incubation.

3.4.4 HBEC-5i binding assay with varying temperatures

Pigmented trophozoites of at least 5% parasitaemia from culture were gelatin purified. The IEs were washed in 10 ml $NaHCO_3$-free DMEM medium. The resulting pellet, of at least 30% parasitaemia, was resuspended at 2% hematocrit and divided equally into three tubes each labelled as room temperature, 37°C or 39°C. Three 48 well plates were also each labelled as room temperature, 37°C or 39°C. For each plate, three wells were seeded with HBEC-5i at least the day before the assay. About 50 ml of $NaHCO_3$-free DMEM medium was put into each of three 50 ml falcon tubes and incubated to room temperature (by leaving it in the hood), 37°C (in a water bath) or 39°C (in an incubator) for at least 20 minutes. The three tubes containing the parasites were centrifuged at 800g for 4 minutes and the supernatant discarded. The cells

were resuspended to 2% haematocrit in 0.1% BSA/$NaHCO_3$-free DMEM medium at the temperature corresponding to the label on the tube.

The HBEC-5i were washed twice with $NaHCO_3$-free DMEM medium (37°C). For each well, 200 µl of the parasite suspension was added per well to the HBEC-5i and the plates incubated at their respective temperatures, room temperature, 37°C and 39°C, for 75 minutes. The plates were gently rocked every 30 minutes and the unbound IEs gently washed off with $NaHCO_3$-free DMEM medium corresponding to their respective temperature. The cells fixed, stained and IEs bound to at least 50 HBEC-5i were counted as previously described.

3.4.5 HBEC-5i binding assay with IEs of varying parasitaemia

Mature pigmented trophozoites were gelatin purified and the parasitaemia obtained was taken as the highest parasitaemia to be tested in that experiment. The IEs were washed and resuspended in $NaHCO_3$-free DMEM medium, pH 7.3, to 2% haematocrit. An aliquot of 500 µl was set aside for the highest parasitaemia for the binding assay and the rest of the parasites were diluted with uninfected erythrocytes to give parasitaemia of approximately 70%, 60%, 50%, 40%, 30%, 20%, 10% and 5%. For experiments where the parasitaemia obtained after gelatin purification gave approximately 60%, or 70%, the parasites were diluted to give the subsequent parasitaemias. For each parasitaemia, the dilution was made from an aliquot of the highest parasitaemia to obtain a final volume of 500 µl and 2% haematocrit. The diluted parasites were thoroughly mixed by repeated resuspension, a smear was made for each and the parasitaemia was determined. The diluted suspensions were centrifuged at 8500g for 7 seconds and the supernatants aspirated. The resulting cells were resuspended to 2% haematocrit in $NaHCO_3$-free DMEM medium.

Prior to the day of the assay, 20 wells of a 48 well plate were seeded with HBEC-5i to reach a confluency of 80 to 100% for the assay. The wells were labelled, 2 wells per parasitaemia to be tested. The HBEC-5i were washed twice

with $NaHCO_3$-free DMEM medium and 200 µl of the parasite suspension added to each of the corresponding wells. The cells were incubated at 37°C for 75 minutes with gentle rocking of the plate every 30 minutes. The cells were gently washed with $NaHCO_3$-free DMEM medium to remove unbound cells, fixed, stained and the number of IEs bound to 50 HBEC-5i was counted as previously described.

3.4.6 HBEC-5i binding assay with serum or plasma

Pigmented trophozoites were gelatin purified to obtain at least 30% parasitaemia and washed twice in $NaHCO_3$-free DMEM medium. The parasites were resuspended in $NaHCO_3$-free DMEM medium to 2% haematocrit. At least a day before the assay, HBEC-5i were seeded on wells of a 48 well plate, with at least 2 wells per test. From the parasite suspension, 500 µl aliquots were dispensed into tubes labelled with the serum to be tested. The no serum control was set aside at 37°C. The rest were centrifuged at 8500g for 7 seconds and the supernatant aspirated. $NaHCO_3$-free DMEM medium and serum (BSA), heat inactivated-FBS, plasma, pooled human serum or heat inactivated pooled human serum from multiple donors with different blood groups (Scottish National Blood Transfusion Service or the Royal Infirmary of Edinburgh, Edinburgh) was added to the suspension to form the desired concentration of either 0.1%, 5% or 10% v/v. The heat inactivated human serum was made by Dr. Ashfaq Ghumra and it involved heating of the pooled human serum at 56°C for 30 minutes to inactivate complement. The suspensions were mixed and 200 µl of each suspension added per well to the HBEC-5i. The cells were incubated at 37°C for 75 minutes with gentle rocking of the plate every 30 minutes. The unbound IEs were gently washed off, the cells were fixed and the number of IEs bound to at least 50 HBEC-5i was counted as previously described.

For experiments investigating the effect of plasma and storage of serum on binding, blood was taken from two individuals (blood types A and B). Five ml of blood was drawn from each individual into 15 ml falcon tubes without an anticoagulant, and a further 5 ml drawn into Acid Citrate Dextrose (ACD, an

anticoagulant) vacutainers on the day of the assay. For serum, the blood was allowed to clot in an upright position for 30 minutes, centrifuged for 15 minutes at 1000g and the supernatant (serum) collected and combined. For the plasma, blood in the ACD tubes was centrifuged for 15 minutes at 1000g and the supernatant (plasma) collected and combined for the binding assay.

3.4.7 HBEC-5i binding assay using MACS or gelatin purified IEs

HB3var03 IEs culture of at least 5% pigmented trophozoites and 1 ml pcv was divided into two equal parts. One half was gelatin purified (section 2.1.5) and the other half was purified by MACS (section 2.7) and used for the binding assay as described in section 2.4.

3.4.8 Flow binding assays with HBEC-5i

A µ-Slide I 0.8 Luer (ibidi, 80191) was coated with 0.1% gelatin for 2 hours and $5x10^5$ HBEC-5i were seeded onto the coated µ-Slide. The cells were incubated at 37°C in 5% CO_2. After 4 hours of incubation, the medium was taken off, to remove unhealthy, dead or unbound cells, and replaced with fresh complete DMEM medium. The cells were incubated with 5% CO_2 at 37°C till they were about 70 to 100% confluent on the day of the assay.

For the assay, IEs at pigmented trophozoite stage were washed twice in $NaHCO_3$-free DMEM medium and made up to 1% haematocrit in 0.1% BSA/$NaHCO_3$-free DMEM medium. The HBEC-5i on the µ-Slide was also washed twice in $NaHCO_3$-free DMEM medium. The ibidi pump system (ibidi, 10902) was set up according to the manufacturer's protocol with the yellow-green perfusion set (ibidi, 10964). Briefly, this involved mounting the perfusion set onto one fluidic unit connected to the ibidi pump, which is attached to a computer with the PumpControl. The software controls the pump and regulates the flow rate, shear stress and shear rate.

The two syringes in the perfusion set were each filled with 5 ml $NaHCO_3$-free DMEM medium and the Luer at the end of each of the two tubing in the

perfusion set, was connected to the opening on the μ-Slide. For each of the syringes, 1.5 ml of the IE suspension was added and the suspension set to flow unidirectional with a switching rate of 5 seconds at a shear stress of 1 dyne/cm^2 and run for 5 minutes. After 5 minutes, the number of IEs bound per 10 fields was counted at x40 on an inverted microscope.

3.5 Results

3.5.1 Environmental factors that can affect adhesion of IEs to HBEC-5i

The effect of the following environmental properties in the host and around the parasite; gaseous conditions, pH, temperature, parasitaemia and serum, on the adhesion of IEs to HBEC-5i was investigated using three DC8 and DC13 -expressing parasite lines (IT4var19 IEs, HB3var03 IEs and IT4var07 IEs) that had been selected for binding to HBEC-5i and also selected by FACS with antibodies to the NTS-DBLα domain of their respective DC13 (HB3var03, IT4var07) and DC8 (IT4var19) PfEMP1 (Claessens *et al.*, 2012). A parasite line, 9197, recently adapted to laboratory culture and selected for HBEC binding (referred to as 9197.HBEC after selection), was also used in some but not all experiments. The major *var* genes transcribed by 9197.HBEC were 9197*var*15 and 9197*var*27 (Chapter 4; section 4.5.3.1).

3.5.1.1 Gaseous conditions

The effect of different gaseous conditions on cytoadhesion was evaluated. The IEs and HBEC-5i were co-incubated in $NaHCO_3$-buffered DMEM medium pH 7.3, under three different gaseous conditions; air (78.09% nitrogen, 20.95% oxygen, 0.93% argon, 0.039% carbon dioxide and other gases), a mixture of 1% O_2 / 3% CO_2 / 96% N_2 (gas used for culturing parasites – referred to as parasite gas) or 5% CO_2 / 95% air (gas for culturing HBEC-5i - referred to as 5% CO_2).

For all four parasite lines tested cytoadhesion was generally highest (though not statistically significant for IT4var07 IEs) in 5% CO_2 which favours

HBEC-5i cultures. DMEM contains sodium bicarbonate to maintain the pH at 7.0 – 7.4; however, sodium bicarbonate is a weak buffer with a pKa of 6.4 hence release of CO_2 and ions from the cells, and changes in atmospheric CO_2 affect the pH of the medium. Approximately 5% of CO_2 is maintained as HCO_3^- in solution, which forms a pH buffer to stabilize the pH as it is in blood *in vivo*. Binding was, hence, significantly reduced ($p < 0.05$) when the culture was incubated in air, (Figure 3.2), which caused the pH to increase to 8.0. There was, however, no significant difference between incubation in the parasite gas, which is hypoxic (1% O_2) and 5% CO_2, which has normal O_2 levels of 20.95%. The pH of the media after incubation in the parasite gas and 5% CO_2 was not measured. Because pH is likely to be a confounding factor in these experiments, further experiments were set up to specifically examine the effect of pH on adhesion of IEs to HBEC-5i.

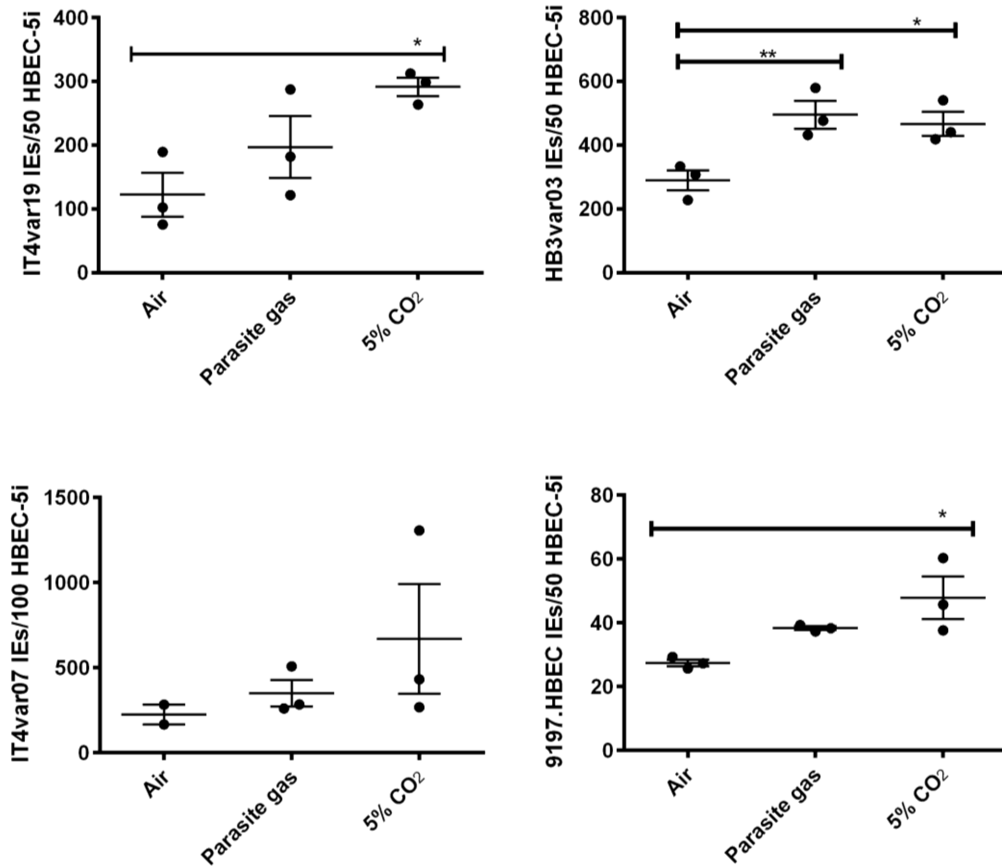


Figure 3.2. Effect of different gaseous conditions on adhesion of IT4var19, HB3var03, IT4var07 and 9197.HBEC IEs to HBEC-5i. Gelatin purified IEs in 0.1% BSA/$NaHCO_3$-buffered DMEM at pH 7.3, of at least 30% parasitaemia, and HBEC-5i were co-incubated in the different gases at 37°C for 75 minutes. Data shown are the mean and SEM of at least two experiments, each with triplicate wells. The log transformed values of the number of IEs bound were compared to each other by repeated measures one-way ANOVA with Tukey's multiple comparisons test (n = 3) with the exception of IT4var07 IEs where comparisons were made using ordinary one-way ANOVA with Tukey's multiple comparisons test because n = 2; *$P < 0.05$. ** $P < 0.01$.

3.5.1.2 pH

The effect of pH on adhesion was investigated via binding assays in air with $NaHCO_3$-free DMEM medium at different pHs. The medium contained HEPES only, a stronger buffer with a pKa of 7.6, which acts as a zwitterion and stably maintains the pH regardless of changes in CO_2 levels. Cytoadherence of IEs to HBEC-5i varied with changes in pH (Figure 3.3). Binding was maximal at pH 7.3 for all four parasite lines. With the exception of IT4var07 IEs, the number of bound IEs significantly increased with increase in pH, from pH 6.8 to 7.3 ($p<0.05$) and began to decrease after pH of 7.3. There were no statistical differences between IEs bound at pH 7.2 and 7.3 for all the four parasite lines. Previous studies had reported cytoadherence to be maximal at pH 6.8, however these were with melanoma cells not brain endothelial cells (Marsh *et al.*, 1988; Sherman and Valdez, 1989; Crandall, Smith and Sherman, 1991).

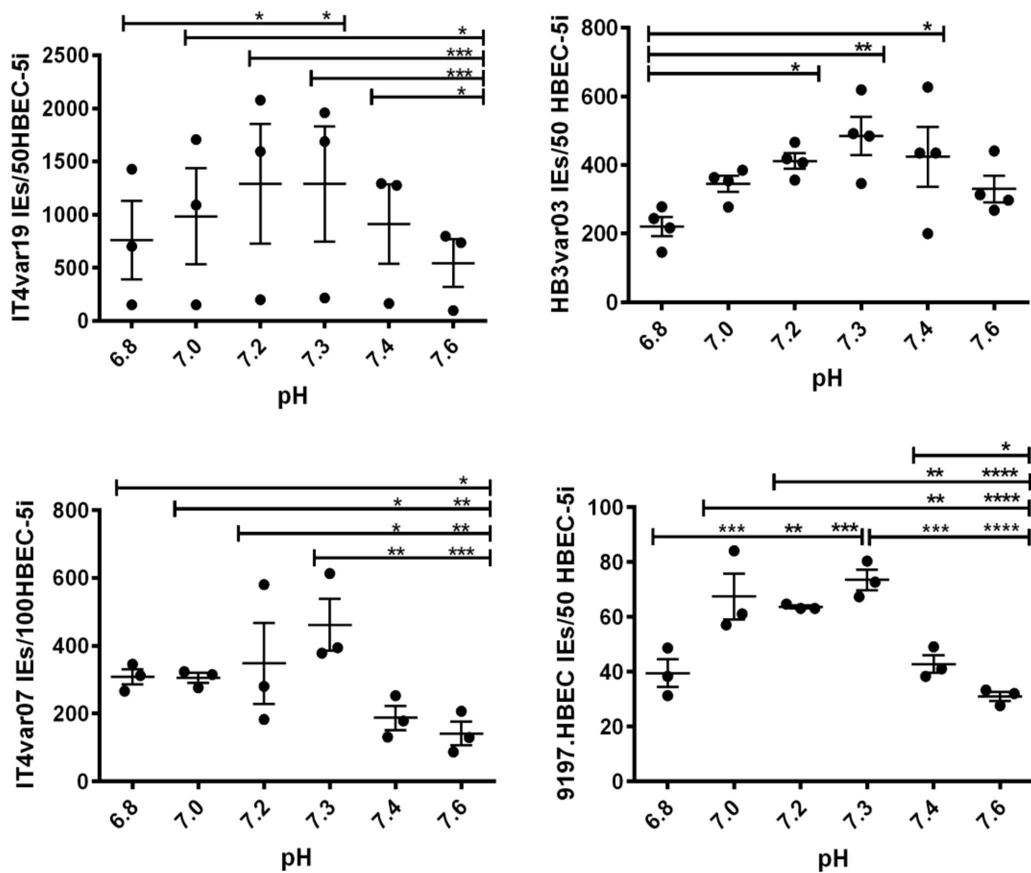


Figure 3.3. Adhesion of IT4var19, HB3var03, IT4var07 and 9197.HBEC IEs to HBEC-5i is maximal at pH 7.3. Binding assays were done with gelatin purified trophozoites (parasitaemia not less than 30%) co-incubated with HBEC-5i in 0.1% BSA/$NaHCO_3$-free DMEM medium at different pHs for 75 minutes. Unbound IEs were also gently washed off with same medium used for the co-incubation. IEs bound to 50 HBEC-5i were counted at 400X magnification. Data shown are the mean and SEM of at least three experiments, each with triplicate wells. P values were obtained from Tukey's multiple comparisons of the log transformed values of the number of IEs bound; ($n \geq 3$) * $P < 0.05$. ** $P < 0.01$, *** $P < 0.001$ and **** $P < 0.0001$.

The pH of the $NaHCO_3$-free DMEM medium in air (Table 3.1) was relatively stable and did not change significantly, unlike that of the $NaHCO_3$-buffered DMEM in air at the end of the binding assay.

pH before binding	pH after binding
6.8	6.73
7.0	6.84
7.2	7.14
7.3	7.27
7.4	7.35
7.6	7.53

Table 3.1. Changes in pH of $NaHCO_3$-free DMEM medium on exposure to air. The media at different pH were incubated in air at 37°C for 75 minutes after which the pH was measured.

To determine if the two buffering systems ($NaHCO_3$-free DMEM medium which contains HEPES only and $NaHCO_3$-buffered DMEM which contains $NaHCO_3$ and HEPES) affected cytoadhesion differently, HB3var03 IEs from the same culture, was divided into two and the binding assay done for each with either $NaHCO_3$-free DMEM medium in air or $NaHCO_3$-buffered DMEM medium in 5% CO_2, both at pH 7.3 and 37°C.

Even though there was the potential change in pH of the $NaHCO_3$-buffered DMEM medium during washes as the medium and the cells were exposed for a relatively short time to air, binding of HB3var03 IEs to HBEC-5i in the two buffering systems in air were similar with no statistical difference, $p = 0.25$ (Figure 3.4). $NaHCO_3$-free DMEM medium was subsequently used for all binding assays, as it was easier to use; a CO_2 incubator was not required.

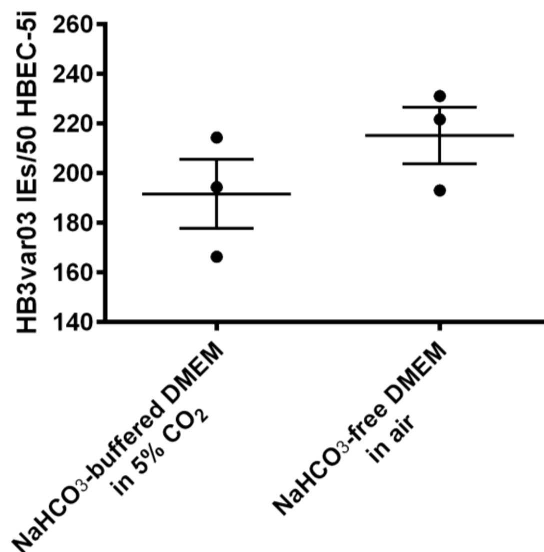


Figure 3.4. Adhesion of HB3var03 IEs to HBEC-5i in two differentially buffered DMEM medium. For the binding assays, gelatin purified IEs from the same culture, of at least 30% parasitaemia, were divided into two; one half was co-incubated with HBEC-5i in 0.1% BSA/$NaHCO_3$-buffered DMEM medium and 5% CO_2, and the other half was co-incubated with HBEC-5i in 0.1% BSA/$NaHCO_3$-free DMEM medium in air. Both media were of pH 7.3 and incubated at 37°C for 75 minutes. IEs bound to 50 HBEC-5i were counted at 400X magnification. Data shown are the mean and SEM of three experiments, each with triplicate wells, n = 3. Comparison was made using a paired t-test; P value = 0.25.

3.5.1.3 Temperature

The effect of different temperature conditions representing low temperature, physiological temperature or fever on cytoadhesion was evaluated by co–incubating the IEs and HBEC-5i at room temperature (16 - 18°C), 37°C and 39°C respectively. The low (room) temperature was tested for experimental reasons.

Similar to previous reports (Sherman and Valdez, 1989; Udomsangpetch *et al.*, 2002), binding of all the parasite lines to HBEC-5i increased with increase in temperature (Figure 3.5), however, no significant difference between cytoadhesion at 37°C and 39°C was observed for all the parasite lines. The increase in cytoadhesion from room temperature to 37 or 39°C was statistically significant for all parasite lines with the exception of HB3var03 and IT4var19,

which did not show a significant increase at 37°C. At room temperature, the HBEC-5i did not look healthy; they looked shriveled or apoptotic. All subsequent experiments were done at 37°C with the exception of the flow experiments which were done at room temperature with medium warmed up to 37°C.

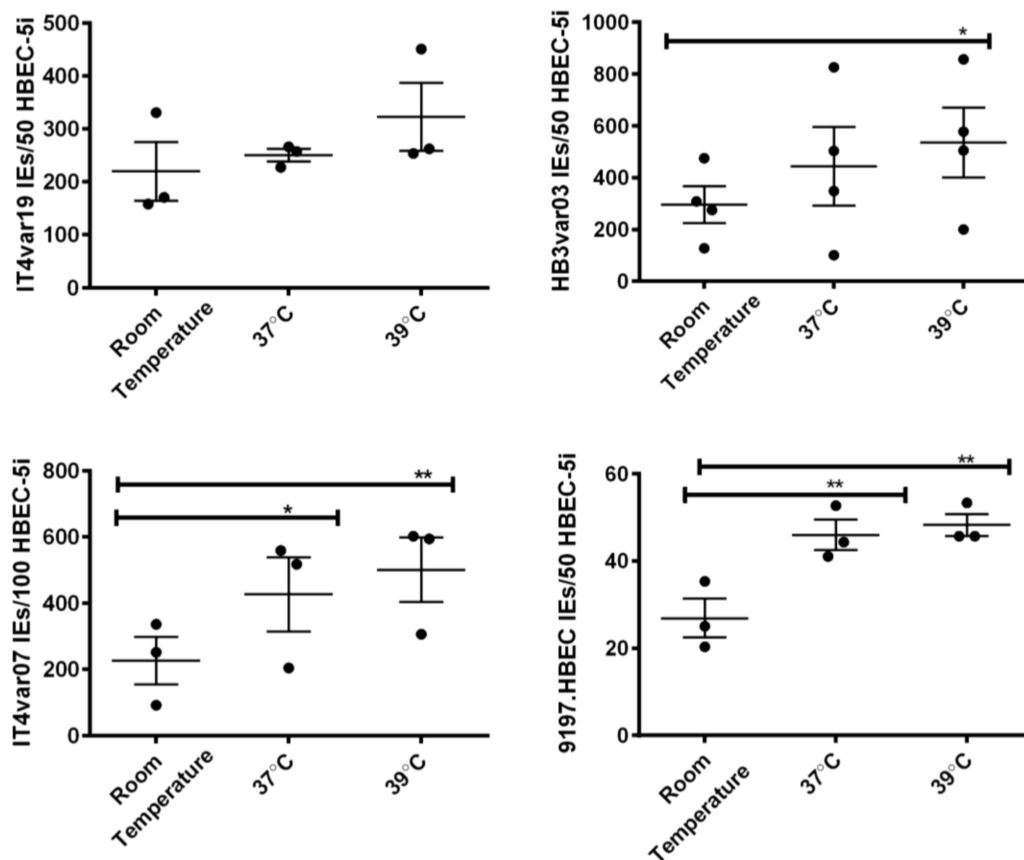


Figure 3.5. Effect of temperature on adhesion of IT4var19, HB3var03, IT4var07 and 9197.HBEC IEs to HBEC-5i. Gelatin purified IEs, of at least 30% parasitaemia, were co-incubated with HBEC-5i at the various temperatures and the unbound IEs were washed off with 0.1% BSA/$NaHCO_3$-free DMEM medium at the respective temperatures. IEs bound to at least 50 HBEC-5i were counted at 400X. Data shown are the mean and SEM of at least three experiments, each with triplicate wells, n $\geq$ 3. Comparisons of log transformed values were made using Tukey's multiple comparisons test in a repeated measures one-way ANOVA; * P value < 0.05 and ** P value < 0.01.

Established standard conditions of 0.1% BSA in $NaHCO_3$-free DMEM medium (DMEM/Nutrient Mixture F-12 Ham with 15 mM HEPES and no $NaHCO_3$), pH 7.3 and incubations at 37°C for 75 minutes in air were used for following experiments unless stated otherwise.

3.5.1.4 Parasitaemia

Purified matured IEs of high parasitaemia (60 to 80%) were diluted with O+ erythrocytes to various parasitaemias over a range of 5% to 80% and static HBEC-5i binding assays were carried out to determine any correlation between parasitaemia and cytoadherence.

At an equal haematocrit of 2%, adhesion to HBEC-5i was dependent on parasitaemia for all three parasite lines with a strong positive correlation; correlation coefficient of 0.96, 0.99 and 0.96 for IT4var19, HB3var03 and IT4var07 IEs respectively (Figure 3.6). The relationship was linear for all three parasite lines, as have been reported for cytoadherence of IEs to melanoma cells (Sherman and Valdez, 1989; Ho and Singh, 1991) and did not saturate at higher parasitaemia as had been previously suggested (Marsh *et al.*, 1988). This data suggests that it is important to use same parasitaemia (at equal haematocrit) or account for differences in parasitaemia when using different parasite lines in comparative studies.

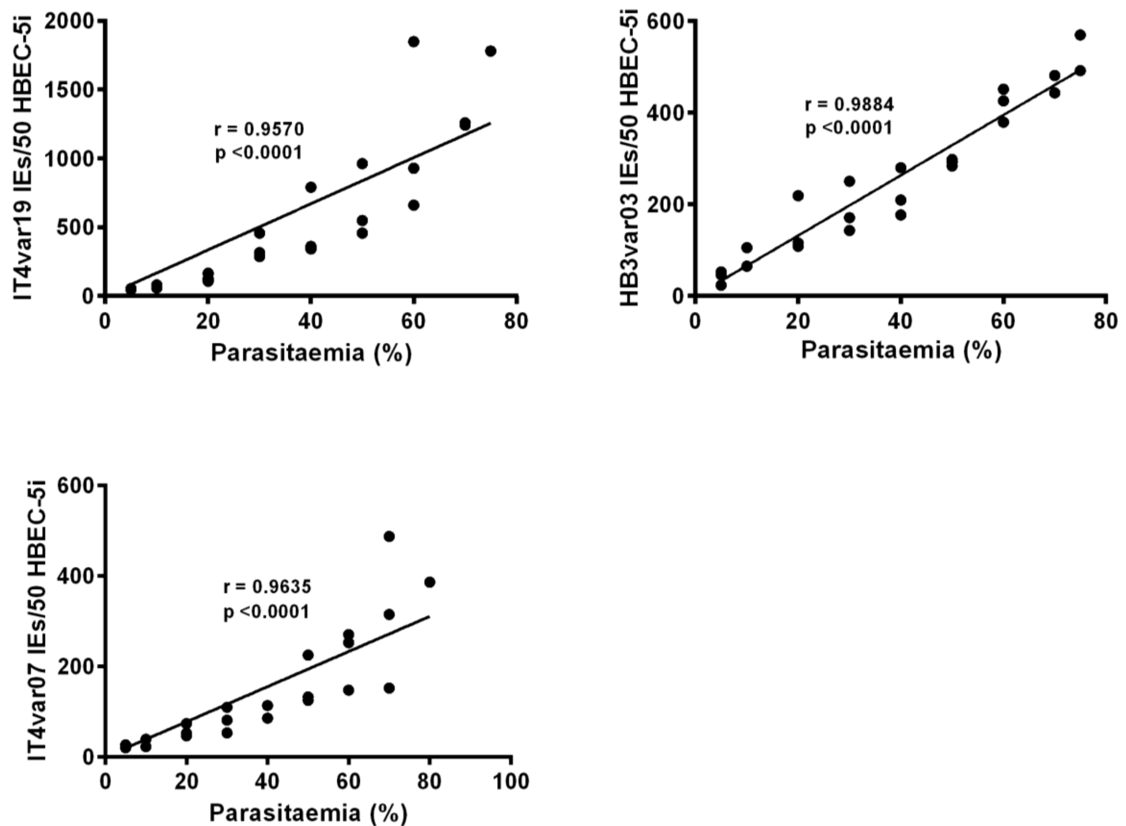


Figure 3.6. Effect of parasitaemia on adhesion of IT4var19, HB3var03 and IT4var07 IEs to HBEC-5i. Gelatin purified IEs were diluted with uninfected erythrocytes to obtain different parasitaemias (5% to 80%) for the binding assay. The IEs in 0.1% BSA/$NaHCO_3$-free DMEM were incubated at 37°C for 75 minutes. IEs bound to 50 HBEC-5i were counted at 400X magnification. A linear regression line is shown for three experiments (n = 3), each done with two wells per parasitaemia tested. The calculated Pearson's correlation coefficient (r) and P value are also shown on the graphs.

3.5.1.5 Serum

All three DC8 and DC13 -expressing parasite lines were selected for HBEC-5i binding in 1% BSA/$NaHCO_3$-buffered DMEM medium without serum (Claessens and Rowe, 2012). Although there have been studies that included human serum in the binding assays (Sherman and Valdez, 1989; Rogerson *et al.*, 1995, 1999), due to the limitations with the availability of serum and the diverse components of serum which makes variations between serum pools a potential problem, in

recent times, parasite binding assays without human serum in the medium are common (Avril *et al.*, 2013; Madkhali *et al.*, 2014; Gillrie *et al.*, 2015). Therefore, to investigate the physiological relevance of these parasites selected on HBEC-5i and the optimal conditions for the binding assay, BSA, pooled human non-immune serum (referred to as 'human serum'), heat inactivated human non-immune serum (referred to as 'HI-human serum') and heat inactivated foetal bovine serum (referred to as 'HI-FBS') were added to the $NaHCO_3$-free DMEM medium at 0.1, 5 or 10% v/v for the HBEC-5i binding assays.

Human serum at 10% significantly inhibited adhesion of all the parasite lines to HBEC-5i (Figure 3.7), however, for IT4var19 IEs, cytoadhesion was completely abolished. The serum inhibition of HB3var03 IEs cytoadhesion confirmed what was previously observed by Claessens (Claessens 2010, PhD). Previous studies with melanoma cells, showed no effect of serum on cytoadhesion, however, the IEs involved were mostly CD36 binders (Sherman and Valdez, 1989; Crandall, Smith and Sherman, 1991). HI-FBS had no effect on adhesion of all the parasite lines. The 0.1% BSA was used to prevent non-specific binding to the HBEC-5i or the plate and did not significantly affect cytoadhesion.

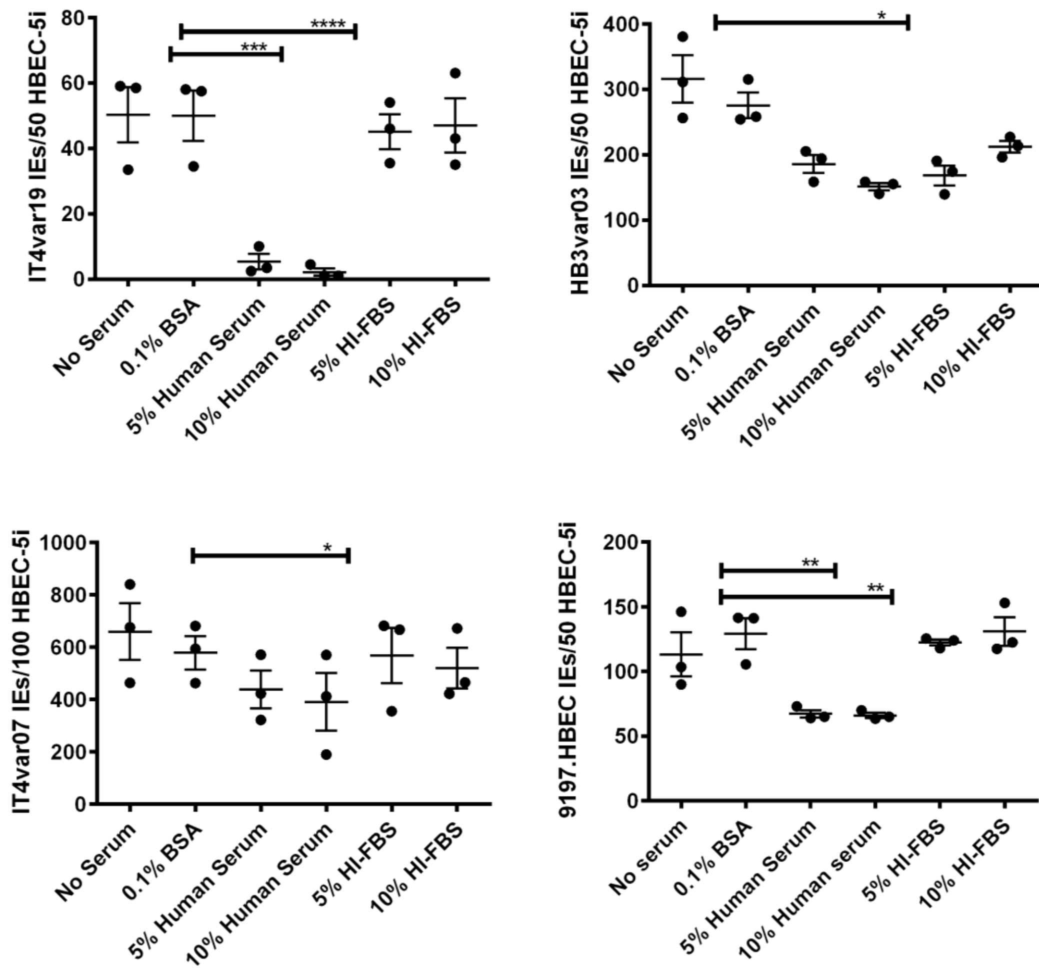


Figure 3.7. Human serum abolishes HBEC-5i adhesion of IT4var19 IEs. Serum was added to gelatin purified IEs of not less than 30% parasitaemia (with the exception of IT4var19 IEs; 5 to 10% parasitaemia was used straight from culture) in $NaHCO_3$-free DMEM medium and the suspension was co-incubated with HBEC-5i and incubated 75 minutes. IEs bound to at least 50 HBEC-5i were counted at 400X. Data shown are the mean and SEM of three experiments, each with duplicate wells. The log transformed means were all compared to that of the 0.1% BSA, using a Dunnet's multiple comparisons test in a repeated measures one-way ANOVA to determine any statistically significant differences; n = 3, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

Heat-inactivated human serum showed similar results, with adhesion of IT4var19 IEs being abolished and no significant effect on the cytoadhesion of IT4var07 and HB3var03 IEs (Figure 3.8). Heating serum inactivates complement and other heat-labile proteins in serum, which may have removed or limited the ability of serum to inhibit cytoadhesion of IT4var07 and HB3var03 IEs. However, since a matched untreated human serum was not tested in the same experiment (in the reported experiments, the batch of serum that was heat inactivated was different from the batch of serum that was not heat inactivated), this effect would have to be investigated further using appropriate matched controls (same batch of serum, heat inactivated and not heated inactivated) to determine the possible role of a heat-labile factor in the serum inhibitory effect on cytoadhesion of the DC13-expressing parasite lines, IT4var07 and HB3var03 IEs.

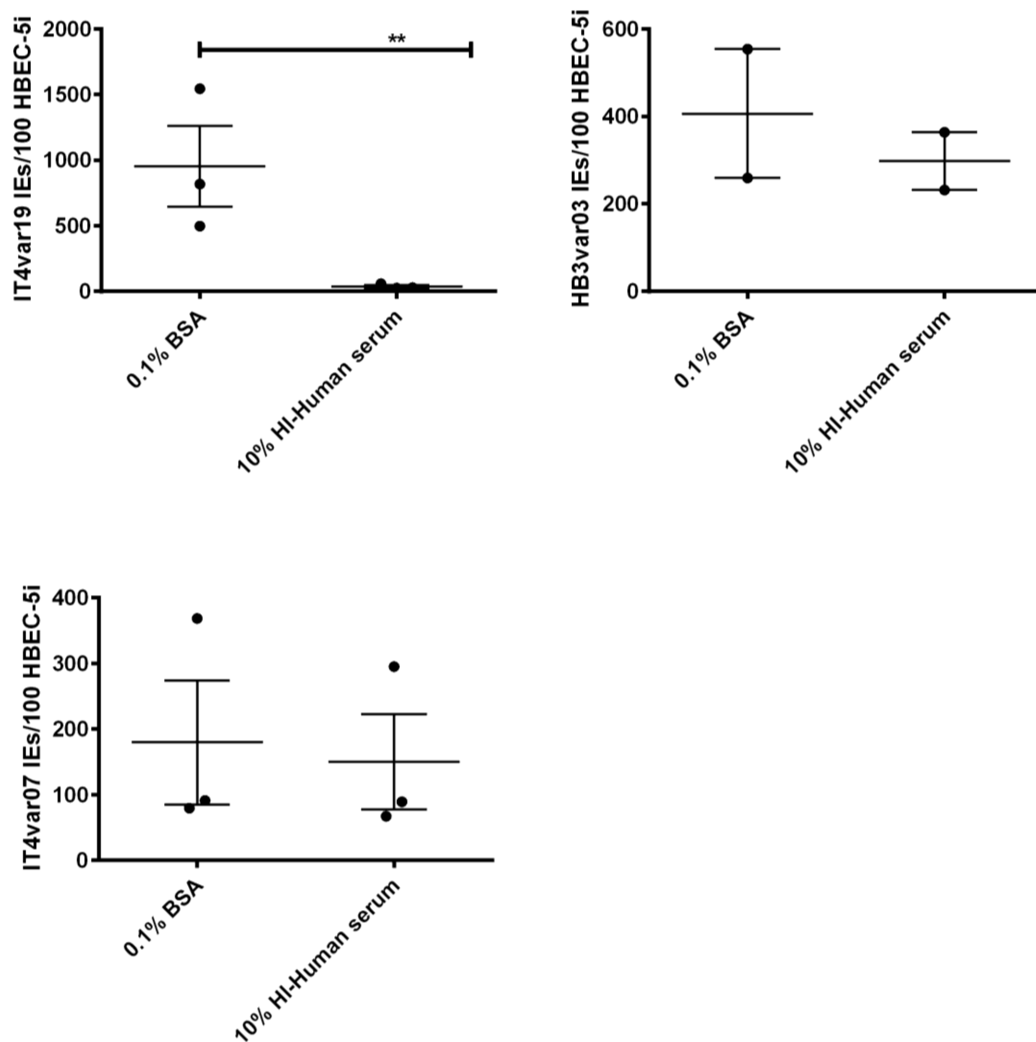


Figure 3.8. Heat inactivated human serum inhibits adhesion of IT4var19 IEs to HBEC-5i. Heat inactivated pooled human serum was added to gelatin purified IEs in 0.1% BSA/$NaHCO_3$-free DMEM medium. The IEs at parasitaemia of at least 30% in the serum were added to HBEC-5i and incubated at 37°C for 75 minutes. IEs bound per 100 HBEC-5i were counted at 400X. Data shown are the mean and SEM of duplicate wells for at least two experiments. Comparisons of the log transformed means were made by a paired t-test; $n \geq 2$, ** $P < 0.01$.

To determine if plasma would affect adhesion differently, binding of IT4var19 IEs to HBEC-5i in 10% v/v plasma in $NaHCO_3$-free DMEM medium, was tested and adhesion was significantly decreased comparable to that of human serum (Figure 3.9). This suggests the inhibitory factor (s) is not part of

the coagulation pathway. Temperature and time of storage could affect the constituents, especially protein content, of human serum (Lee *et al.*, 2010; Cuhadar *et al.*, 2013). The human serum was usually stored at -20°C or -70°C hence it is possible that changes in the stored serum caused the inhibition of IT4var19 IEs binding to HBEC-5i. The blocking of IT4var19 IEs adhesion to HBEC-5i was, however, not affected by storage of the serum, as freshly collected serum (used within 24 hours of collection) and stored serum gave similar results.

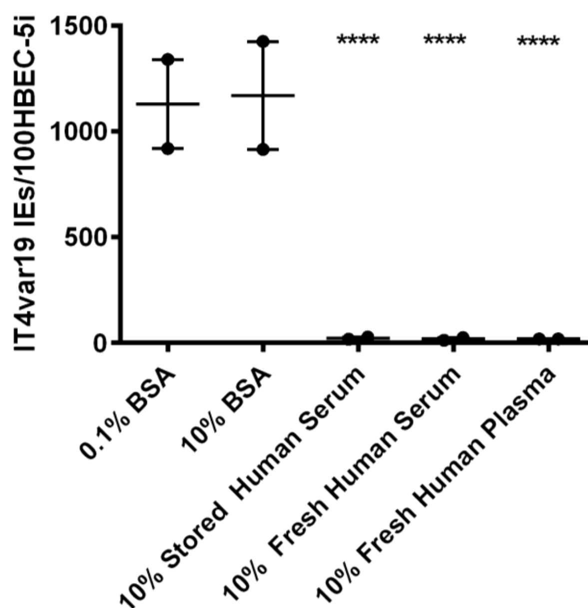


Figure 3.9. Plasma and serum abolish adhesion of IT4var19 to HBEC-5i. Human plasma and serum were obtained from two donors and used for the binding assays within 24 hours. Human serum, which had been previously frozen, was also used for comparison to the fresh serum. Plasma or serum was added to gelatin purified IEs (parasitaemia of at least 30%) in DMEM binding medium and co-incubated with HBEC-5i. IEs bound per 100 HBEC-5i were counted at 400X. Data shown are the mean and SEM of two experiments, each with duplicate wells. The log transformed means were compared to that of the 0.1% BSA control in a Dunnett's multiple comparisons test with repeated measures one-way ANOVA of the log transformed means; n = 2, **** $P < 0.0001$.

3.5.2 Effect of gelatin purification for IEs with knobs on binding to HBEC-5i

Adhesion of two differentially purified mature IEs to HBEC-5i were compared. IEs from a culture of predominately mature pigmented trophozoites was divided into two. One half was purified by MACS (which purifies pigmented trophozoite IEs, irrespective of the presence of knobs) and the other half was purified by gelatin flotation (which enriches for knob-positive pigmented trophozoite IEs). Adhesion of both differentially purified IEs to HBEC-5i was tested. Parasitaemia of IEs purified by MACS was 80% and 88% while that of the gelatin purified IEs was 67% and 75% respectively for the two experiments.

The gelatin purified IEs gave similar results as the MACS purified IEs with respect to binding to HBEC-5i (Figure 3.10). The parasites lines used here routinely underwent gelatin purification at least once a month, therefore the similar binding profile of both MACS and gelatin purified IEs may have been due to an equal number of knob-positive IEs in both cultures. However, to investigate the effect of knobs on adhesion in more detail, the expression of KAHRP by the IEs would have to be determined before binding to HBEC-5i.

This experiment does suggest that the use of gelatin for the purification of pigmented trophozoites IEs with knobs had no deleterious effect on the binding of the IEs to HBEC-5i. Subsequent binding experiments were mostly done with gelatin purified IEs, not only for IEs with knobs but to increase the parasitaemia so that any differences between samples within experiments would be clearly obvious when measured, and also because it is simpler, and less time-consuming than MACS.

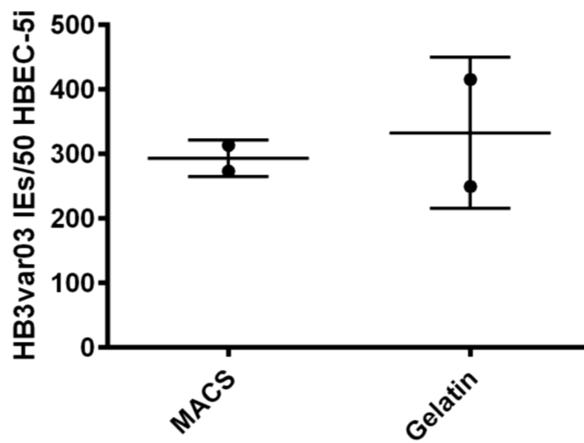


Figure 3.10. Effect of MACS and gelatin purification on adhesion of HB3var03 IEs to HBEC-5i. From the same culture, pigmented trophozoites in half of the culture was gelatin purified and the other half of the culture underwent MACS, also for purifying pigmented trophozoites. The purified IEs were each co-incubated with HBEC-5i for 75 minutes and IEs bound per 50 HBEC-5i counted at 400X. Data shown are the mean and SEM of two experiments with duplicate wells. There was no significant difference between the two purification methods; n = 2, P > 0.05, from a paired t-test.

3.5.3 HBEC-binding IEs also bind under flow conditions

IEs bind to host cells during blood perfusion when it is flowing through the microvasculature. While *in vitro* flow binding assays may be physiologically relevant in mimicking perfusion, static binding assays have been widely used for adhesion assays as they are relatively easier and cheaper to do. The four parasite lines used, HB3var03, IT4var7, IT4var19 and 9197.HBEC were selected for adhesion to HBEC-5i under static conditions. To ascertain whether these parasites would also bind to HBEC-5i under flow, and hence their physiological relevance, a flow-binding assay was done at a physiological flow rate of 1dyne/s using HB3var03, IT4var7 or IT4var19 IEs.

Cytoadhesion of IEs under static conditions may differ from that under flow as differences in binding to ICAM-1 had been observed between parasite lines under flow and static conditions (Gray *et al.*, 2003; Ochola *et al.*, 2011), and for CD36 binding, IEs that bind under static conditions may require knobs for

binding under flow (Crabb *et al.*, 1997). All three parasite lines (regularly gelatin-purified in culture to maintain knobs on the surface of the IEs) bound to HBEC-5i under flow (Figure 3.11). This was in agreement to what was reported by (Gillrie *et al.*, 2015) who showed that their IT4var07 and IT4var19 IEs they had selected in their laboratory also bound to transformed human brain microvascular endothelial cells (THBMEC) under flow. For the three parasite lines tested, IEs that bound, were static on contact with the HBEC-5i and did not tether or roll.

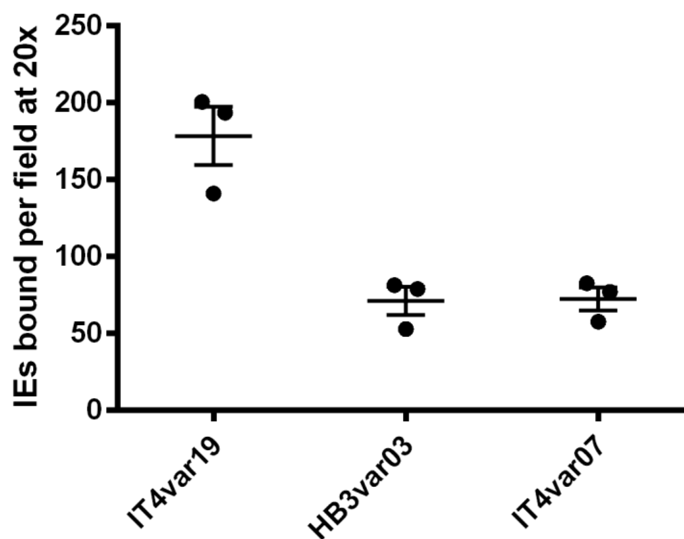


Figure 3.11. IT4var19, IT4var07 and HB3var03 IEs also bind to HBEC-5i under flow conditions. Gelatin purified IEs, of at least 30% parasitaemia, were directed over HBEC-5i on a μ-slide under parallel flow conditions at shear stress of 1 dyne/cm^2. Number of bound IEs were counted for ten fields at 200X. The data shown are mean and SEM of an average of 10 fields counted for each of three independent experiments (n = 3).

3.6 Discussion

It is important to investigate the mechanisms involved in the cytoadherence of IEs to HBEC, to elucidate the pathology of sequestration in cerebral malaria. Knowledge about conditions optimal for binding, and effect of the environment are useful for *in vitro* assays and could also provide insight into factors that can affect cytoadhesion *in vivo*.

Hypoxia, which is associated with cerebral malaria, has been reported to cause an increase in expression of adhesion receptors, such as ICAM-1, on endothelial cells (Antonova *et al.*, 2009) however other studies observed no such increase in receptors under hypoxic conditions (Maurus *et al.*, 2003; Ziegelstein, He and Hu, 2004). Binding of IEs to HBEC in normoxia was similar to cytoadhesion in hypoxic conditions. Cytoadhesion was, however, significantly reduced in low CO_2 (0.003%) compared to 5% CO_2 and this may have been due to changes in pH (an increase of 7.3 to 8.0) of the $NAHCO_3$-buffered medium in the low CO_2 concentration.

Cytoadherence of the HBEC-binding parasites was generally maximal at pH 7.3, close to physiological pH and was similar for all four parasite lines. The pH binding profile was quite similar to that of CSA (Pouvelle *et al.*, 1998; Beeson and Brown, 2004) but unlike that of CD36 which binds optimally at acidic pH between 6.6 and 6.9 (Marsh *et al.*, 1988; Crandall, Smith and Sherman, 1991). Blood pH has been shown to be on average 7.16 for children with cerebral malaria and respiratory distress, and 7.39 for those with cerebral malaria only (English *et al.*, 1997); cytoadhesion to HBEC was not significantly affected by changes in pH within this range. As it has been suggested for CSA (Pouvelle *et al.*, 1998), it is possible that these DC8 and 13 -expressing IEs, bind to the endothelial cells in the microvasculature and obstruct blood flow, creating an acidic environment conducive for binding of other IEs to CD36 to increase sequestration in other tissues. For the assay, optimal pH of 7.3 is recommended as both parasites and endothelial cells also grow well at this pH.

There have been several studies on the effect of febrile temperatures on parasite growth but few studies have looked at temperatures changes in

relation to cytoadherence. Previous published work examined adhesion to host receptor recombinant proteins or transfected fibroblasts and reported an increase in cytoadherence to ICAM-1 and CD36 at febrile temperature of 40°C (Udomsangpetch *et al.*, 2002). However, although binding of all the parasite lines to HBEC increased with increase in temperature from 37°C to 39°C, this was not statistically significant in this study. The differences in results may be due to the different temperatures tested and the type of cells used. Febrile temperature induced increase in binding has been attributed to an increase in expression of PfEMP1 by the matured trophozoites (and rings) (Udomsangpetch *et al.*, 2002) or increase in rigidity of matured IEs (Marinkovic *et al.*, 2009), which may impede or restrict flow through the microvasculature and promote binding to the endothelial cells. However, in another study with a different parasite strain, there was no increase in the expression of PfEMP1 (Oakley *et al.*, 2007; Carvalho *et al.*, 2013). On the host side, febrile temperatures had no effect on expression of adhesion receptors (including ICAM-1, PECAM-1, VCAM-1 and P- selectin) on unstimulated human microvascular endothelial cells (Shah *et al.*, 2002) and had no effect on binding of neutrophils to endothelial cells (Hasday *et al.*, 2001). It has also been noted that clinically, brain and body temperature in cerebral malaria did not differ from that of uncomplicated malaria cases and were observed to be within the range of 37 to 39°C (Esamai *et al.*, 2001). There were no statistical differences in cytoadhesion within this range. For the binding assays, physiological temperature, 37°C would be the optimal temperature to use as temperatures below or above that may lead to adverse effects on the IEs or the brain endothelial cells.

A positive correlation between parasitaemia and adhesion to HBEC was seen with all the parasite variants and was also consistent with similar studies which examined binding of IEs to melanoma cells (Marsh *et al.*, 1988; Sherman and Valdez, 1989; Ho and Singh, 1991). This result is important for the *in vitro* binding assay, as parasitaemia can be increased to optimize the assay and it also shows that it is crucial to control for differences in parasitaemia for comparative cytoadherence studies.

Serum has been shown to be important for the presentation of PfEMP1 on the surface of IEs, and also promote or enhance binding to host receptors proteins (Frankland *et al.*, 2007; Tilly *et al.*, 2015). The parasites used for this study were cultured in a mixture of both serum and albumax (section 2.1.2). The inhibitory effect of serum on IT4var19 IEs suggests that there may be a serum factor that either binds to the surface of the IEs or the receptor on the HBEC-5i at or close to the binding site. The ability of serum to abolish adhesion of IT4var19 IEs, a DC8 parasite, to HBEC-5i, suggests that it may be binding to a different receptor than the other two DC13 parasites, and that IT4var19 IEs adhesion to HBEC-5i may not be physiologically relevant.

In addition to serum, another factor of physiological relevance is binding under flow. Due to the cost and complexity of flow binding assays, static binding assays are the most commonly used. Cytoadherence of IEs to certain receptors that bind under static conditions have been shown to be significantly reduced under flow (Crabb *et al.*, 1997). It is therefore important to investigate whether the parasites lines used in this thesis can bind to HBEC-5i under flow rates that mimic physiological conditions. Similar to what has been reported for rosettes (Adams and Rowe, 2013), the parasites that were selected for binding to HBEC-5i under static conditions also bind under flow. This confirmed what others have shown previously (Phiri *et al.*, 2009; Gillrie *et al.*, 2015).

Changes in the environmental conditions may regulate cytoadhesion by modifying the IEs, the HBEC or both. The surface of both IEs and HBEC consists of proteins, glycosaminoglycans, glycolipids and proteoglycans, which can be affected by changes in environmental conditions such as pH and temperature. Effect of changes in gaseous conditions, pH and temperature could lead to the direct modification of the expression of PfEMP1 or modify other molecules implicated in the presentation and binding of PfEMP1 such *Plasmodium* helical interspersed subtelomeric (PHIST) proteins (Oberli *et al.*, 2016) which may affect cytoadhesion. Differences in expression of surface receptors such as ICAM-1 and VCAM have been reported with regards to changes in environmental conditions such as acidosis and pH, (Antonova *et al.*, 2009), it is

possible EPCR and other receptors involved in the adhesion of the HBEC-binding IEs may be modified by changes in the environmental conditions.

To conclude, the conditions for optimal binding to HBEC was similar for all the parasites lines with regards to gaseous conditions, pH and temperature, however, there were differences in the binding properties in human serum, which abolished binding of only IT4var19 IEs. This may suggest that the host receptor and parasite ligand interactions involved in the binding of IT4var19 (DC8-expressing) may be different from that of HB3var03 and IT4var07 (DC13-expressing) parasite lines. Although this study has thrown light on the binding properties of four *P. falciparum* parasite lines that bind to brain endothelial cells and shown which conditions are optimal for *in vitro* binding assays, further studies with more replicates, clinical isolates and more DC8 and 13 -expressing parasites lines are needed to generalize these observations.

4 Chapter IV: *Var* gene profiling of *P. falciparum* infected erythrocytes that bind HBEC-5i and investigation of the PfEMP1 domain mediating adhesion

4.1 Abstract

P. falciparum express variant surface antigens, PfEMP1 (s) encoded by v*ar* genes, on the surface of infected erythrocytes (IEs) that are thought to enable IEs to sequester by binding to host cells to avoid splenic clearance. Sequestration of IEs in the microvasculature of the brain contributes to the pathology of cerebral malaria. Parasites that express group A or B/A PfEMP1 containing domain cassettes (DC) 8 and 13 have been associated with adhesion to brain endothelial cells and suggested to bind to EPCR on the host cells by the CIDRα1 domain of the PfEMP1. Two Kenyan isolates, 8211 and 9197, recently adapted to culture, were selected for binding to HBEC-5i and found to predominantly transcribe group A-like *var* genes that encode DBLα1.2 or DC8 PfEMP1, after selection. Recombinant proteins and antibodies to DC8 and DC13 domains of three parasite lines expressing variants HB3var03, IT4var07 and IT4var19 that bind HBEC-5i, were also used in binding assays to determine which domain(s) was mediating cytoadhesion. It is shown here that with the exception of adhesion of the EPCR-binding strain, IT4var19 that was inhibited by the CIDRα1 domain of all three parasites, the CIDRα1 and DBLβ recombinant proteins tested had no effect on cytoadhesion. Antibodies to the NTS.DBLα1 inhibited adhesion of all the DC8 and DC13 -expressing IEs. These data confirm the association of a subset of group A and B/A *var* genes with binding to HBEC, shows that DBLα1.2-CIDRα1 domain cassettes (non- DC8 and DC13) are also expressed by HBEC-binding IEs and suggest that multiple PfEMP1 domains may be involved in cytoadhesion.

4.2 Introduction

Individuals living in malaria endemic areas tend to develop immunity to severe disease early in life but an estimated 10-12% of children develop severe malaria before the onset of this protection from disease (Gonçalves et al. 2014). The most virulent malaria parasite in humans is *P. falciparum* and perhaps it is so because of its variant surface antigens encoded by *var* genes. The ability of the parasite to express any one of the 50-60 *var* genes at a time is thought to enable to parasite to sequester by binding to host cells to avoid splenic clearance resulting in obstruction of the microvasculature and increased virulence of the disease (Smith *et al.,* 1995; Rowe *et al.,* 2009).

Var genes encode PfEMP1, the ligand implicated in the sequestration of IEs in microvasculature of the host, a feature in the brain peculiar to the pathophysiology of cerebral malaria. (MacPherson *et al.*, 1985; Taylor *et al.*, 2004; Milner *et al.*, 2014). *Var* genes and hence the PfEMP1 variants can be classified into groups A, B, C and E depending on their upstream sequence, location in the chromosome and direction of transcription (Gardner *et al.*, 2002; Kraemer and Smith, 2003; Lavstsen *et al.*, 2003).

PfEMP1 molecules consist of an N-terminal segment (NTS), Duffy binding-like domains (DBL) and cysteine rich interdomain regions (CIDR) domains followed by a transmembrane (TM) region and an acidic terminal segment (ATS) (Baruch *et al.*, 1997; Smith *et al.*, 2000a; Hviid and Jensen, 2015). Binding of the extracellular DBL and CIDR domains to host receptors has been widely described. Examples include binding of CIDRα2 to CD36 (Baruch *et al.*, 1997), DBLβ to ICAM-1 (Smith *et al.*, 2000b) and DBL1α to heparan sulfate (Angeletti *et al.*, 2015). PfEMP1 variants can also be classified in terms of domain cassettes (DC) based on characteristic sets of the DBL and CIDR domains occurring together and the conservation of these domain cassettes may have functional relevance (Rask *et al.*, 2010).

The organ-specific nature of cerebral malaria and its associated sequestration in the brain suggest that parasite expression of specific PfEMP1 variants that can bind to HBEC may be pertinent to the pathology of the disease.

Several studies have shown evidence of higher transcription of group A *var* genes in severe malaria isolates relative to isolates from children with uncomplicated disease, which predominantly transcribe group B and C *var* genes (Kirchgatter and Portillo, 2002; Jensen *et al.*, 2004; Kyriacou *et al.*, 2006; Rottmann *et al.*, 2006; Lavstsen *et al.*, 2012; Mugasa *et al.*, 2012). Other studies have, however, also reported higher transcription of group B *var* genes in the severe malaria isolates (Kaestli *et al.*, 2006; Falk *et al.*, 2009; Kalmbach *et al.*, 2010). Parasites that transcribe group A-like DC8 and DC13- encoding *var* genes (the domain architecture is illustrated in Figure 4.1, have been associated with cerebral malaria (Bertin *et al.*, 2013; Almelli *et al.*, 2014b; Tembo *et al.*, 2014). These same DC8 and DC13 -expressing variants have been shown to bind to HBEC (Avril *et al.*, 2012; Claessens *et al.*, 2012) and the CIDRα1 domain of the DC8 and DC13 PfEMP1 also shown to bind to EPCR, a receptor on HBEC (Turner *et al.*, 2013; Lau *et al.*, 2015).

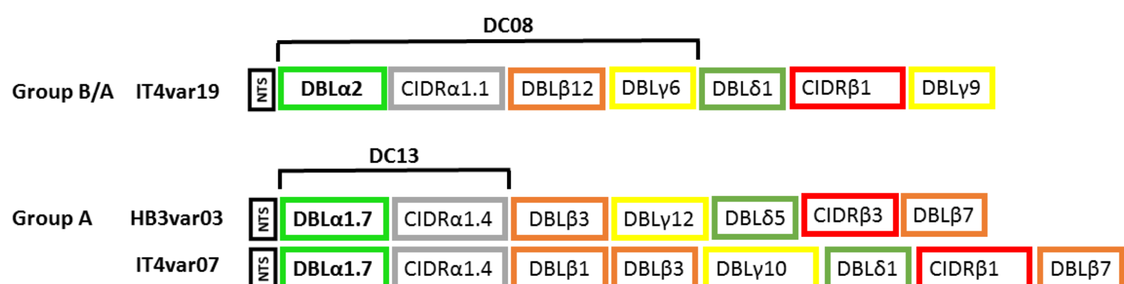


Figure 4.1. PfEMP1 domain architecture of IT4var19, HB3var03 and IT4var07. The Group B/A (DC08) PfEMP1 are encoded by *var* genes with an upstream B sequence and transcription is towards the centromere. They have the N-terminal DBLα2 of group B and the domain structure of group A PfEMP1.

The ability of the group A and B/A, DC8 and DC13 -expressing parasites to cytoadhere to human brain endothelial cells has so far been based on three laboratory adapted parasites lines, 3D7, HB3 and IT4 (DC8 and DC13 *var* genes identified were 3D7 PFD0020c, HB3*var*03, IT4*var* 06, 07, 19 and 20 respectively) (Avril *et al.*, 2012; Claessens *et al.*, 2012; Turner *et al.*, 2013). It is

therefore important to investigate more parasite lines, especially clinical isolates to confirm this association and to investigate the role of the DC8 and DC13 domains in mediating cytoadhesion to HBEC.

4.3 Hypothesis and specific aims

In this chapter, the hypothesis that IEs, which bind to HBEC, express group A or B/A PfEMP1 containing DC8 and DC13, and that the binding is mediated by the CIDRα1 domain of these DC8 and DC13 PfEMP1, was investigated.

Specific aims

1. To select three Kenyan *P. falciparum* isolates recently adapted to culture, SAO75, 9197 and 8211, for binding to HBEC-5i
2. To identify the predominantly transcribed *var* gene in the HBEC-5i-selected SAO75, 8211 and 9197 parasites.
3. To determine which domain (s) of the DC8 and DC13 PfEMP1 mediates adhesion to HBEC-5i.

4.4 Methods

4.4.1 Immunofluorescence assay for detection of knobs on the surface of IEs

Thin blood smears were made from approximately 10 µl suspension of parasite culture of mature pigmented trophozoites at not less than 5% parasitaemia and air-dried. The smears were fixed with cold 90% acetone/10% methanol for 30 minutes. After air drying the smears, two circles were drawn on each slide with nail varnish to form wells, dried and 500 µl of 1/1000 dilution of knob-associated histidine-rich protein (KAHRP) monoclonal antibody raised in mouse, mAb 89 (Taylor *et al.*, 1987) or mouse IgG in PBS/1% BSA was added to each well. A 'no antibody' control of 500 µl PBS/1% BSA was also added to one

of the wells. The slides were incubated for 1 hour at room temperature in a humid box.

After incubation, the slides were tipped to allow the antibodies to run off and washed three times by immersion in a staining jar filled with 1X PBS, for 5 minutes, after which the PBS was poured off and replaced with fresh PBS for the next wash. For the secondary incubation, 500 µl of 1 /500 Alexa Fluor 488 highly cross-absorbed goat anti-mouse IgG (Thermo Fisher Scientific, A11029) in PBS/1% BSA/1 µg/ml DAPI was added to each slide and incubated for 45 minutes at room temperature in a humid box. The slides were washed three times as described above, air-dried and each mounted with a drop of 1.25 mg/ml of DABCO in 50% glycerol/50% PBS. A coverslip was placed on the mounted slides and the edges sealed with nail varnish. The number of IEs, positively stained for KAHRP, out of 100 IEs in at least 4 fields were counted at 1000X with a fluorescence microscope and pictures were taken with a YenCam 5-megapixel camera and YenCam imaging software (both from Microscope Systems Limited) attached to the microscope and a computer (Dell).

4.4.2 Selection of *P. falciparum* isolates on HBEC-5i

At least, a day before the assay, HBEC-5i were seeded onto a 0.1% w/v gelatin in dH_20 coated T75 cm^2 flask, to reach 80-100% confluency on the day of the assay. For the assay, mature parasites at the pigmented trophozoite stage and of at least 5% parasitaemia were washed twice with DMEM binding medium. For the washes, the parasite culture was centrifuged at 800g for 4 minutes, the supernatant aspirated and the pellet made up to 2% haematocrit with DMEM binding medium. The suspension was centrifuged at 800g for 4 minutes and the wash repeated with DMEM binding medium. The pellet was made up to 2% haematocrit in 0.1% BSA/DMEM binding medium. HBEC-5i was also washed twice with DMEM binding medium, by adding the medium, swirling it over the adherent cells, aspirating off the medium and repeating the wash. The parasite suspension was added to the HBEC-5i and co-incubated for 75 minutes at 37°C with gentle resuspension of cells after 30 and 60 minutes of incubation.

The unbound cells were washed off, by gently rocking the flask in four directions, clockwise and anticlockwise before the medium was gently taken off and replaced with fresh DMEM binding medium. The washes were repeated and the flask checked between washes under an inverted microscope until there were few or no unbound cells. Approximately, 100 µl of fresh erythrocytes at 50% haematocrit was added to the IEs bound to HBEC-5i and incubated in complete RPMI medium (described in chapter 2.1.2) overnight in a mixture of 1% O_2, 3% CO_2 and 96% N gas at 37°C. As schizont stage parasites burst, merozoites reinvaded the erythrocytes and the ring stage parasites were harvested the following day. Parasites were cultured until enough material was obtained (typically 2 to 4 weeks) and the level of binding to HBEC-5i assessed after at least 3 rounds of panning. The panning was repeated if required, until the level of binding to HBEC-5i increased to appreciable levels.

4.4.3 Molecular analysis to determine *var* gene expression of IEs

4.4.3.1 Extraction of RNA

RNA was extracted from IEs at the late ring stage when *var* gene mRNA levels are high (Kyes, Pinches and Newbold, 2000; Dahlbäck *et al.*, 2007) and at a parasitaemia of at least 5%. The culture in a sterile 15 ml falcon tube was centrifuged at 800g for 4 minutes and the supernatant, aspirated. A volume of TRIzol (Thermo Fisher Scientific, 15596026) 10 times the pcv of the culture was added to the pellet and mixed until the clumps dissolved. The suspension was incubated at 37°C for 5 minutes and stored at -70°C until the extraction was continued. The suspension was thawed to room temperature and chloroform (Sigma-Aldrich, C2432) (0.2 times the original TRIzol volume) was added. The suspension was shaken vigorously for 15 seconds and left to stand at room temperature for 2 minutes after which it was centrifuged at 3600g for 40 minutes at 4°C.

The suspension separated into three layers, an organic phase at the bottom, an interphase and an aqueous phase at the top containing the RNA. The

aqueous phase was gently transferred to a new 15 ml falcon tube and isopropanol (VWR, 20847.373) (0.5 times the original TRIzol volume), added. The suspension was gently mixed, by inverting the tube a few times. The suspension was incubated at 4°C for at least 2 hours or overnight, vortexed and centrifuged at 3600g for 60 minutes at 4°C.

The supernatant was pipetted out and the RNA pellet washed in 5 ml of 75% ethanol (VWR, 20821) in 0.1% diethylpyrocarbonate (DEPC) in ddH_2O (DEPC-H_20). The suspension was centrifuged at 7500g for 5 minutes at 4°C. The supernatant was gently removed and the pellet, air-dried for 5 minutes. The pellet was dissolved by resuspension in 50–100 µl of DEPC-H_20 pre-warmed to 60°C and heated at 60°C for 10 minutes before it was transferred to ice. The RNA was transferred to RNAse free Eppendorf tubes and its concentration and purity determined using the NanoDrop 1000 (Thermo Fisher Scientific). The RNA was stored at -70°C.

4.4.3.2 cDNA preparation from extracted RNA

Contaminating genomic DNA in the extracted RNA was removed before reverse transcription by adding 1.5 µl each of DNAse 1 and 10X DNAse buffer (200 mM Tris-HCl pH 8.4, 20 mM $MgCl_2$, 500 mM KCl) (Thermo Fisher Scientific, 18068-015) to 2 µg RNA**.** The reaction mix was made up to 15 µl with ddH_2O and incubated at room temperature for 5 minutes after which 1.5 µl of 25 mM EDTA (Thermo Fisher Scientific, 18068-015) was added to prevent the degradation of the RNA during heat inactivation of the DNAse enzyme. The enzyme in the reaction mix was inactivated by heating the sample at 65°C for 10 minutes.

Two tubes were set up with 1 µg of RNA per tube and designated as "plus RT" and "no RT", the latter being a control to detect any genomic DNA contamination in the Reverse Transcriptase PCR (RT-PCR). For each tube, 5 ng/µl of random hexamers and 1 mM deoxyribonucleotide triphosphate dNTP mix (Thermo Fisher Scientific, 18080-051) was added to the 1 µg RNA (8 µl RNA from above) in a total volume of 10 µl. The reaction mix for both were incubated at 65°C for 5 minutes and put on ice for 2 minutes after which it was centrifuged

at 3600g for 4 seconds. To both tubes, 1X Reverse Transcriptase (RT) buffer (200 mM Tris-HCl pH 8.4, 500 mM KCl), 5 mM $MgCl_2$, 0.01 M Dithiothreitol (DTT) and 2 U/µl RNAseOUT ribonuclease inhibitor (all from Thermo Fisher Scientific, 18080-051) were added to the reaction mix. One U/µl of Superscript III Reverse Transcriptase (Thermo Fisher Scientific, 18080-044) was added to the "plus RT" sample and 1 µl of 0.1% DEPC/H_2O to the "no RT" control in a total volume of 20 µl each for both tubes. The reaction mix was gently mixed, centrifuged at 3600g for 4 seconds and put on the PCR machine, DNA Engine Peltier Thermal Cycler PTC-0200 (Bio-Rad) with the following settings; incubation at 25°C for 10 minutes, followed by a 50-minute incubation at 50°C and an increase in temperature to 85°C for 5 minutes. The samples were placed on ice and used immediately or stored at -20°C until used in PCR.

4.4.3.3 Amplification of DBLα sequence tags

A semi-conserved region of 300 to 500 base pairs of the DBLα domain was amplified by RT-PCR, using degenerate primers, αAF' - 5' GCACG(A/C)AGTTT(C/T)GC and αBR - 5' GCCCATTC(G/C)TCGAACCA) (Bull *et al.*, 2005). Two tubes labelled as "plus RT" or "no RT" (to correspond with the samples in section 4.4.3.2) were each set up with a total volume of 50 µl containing 2 mM $MgCl_2$, 1.25 mM dNTPs, 50 pmols each of αAF' and αBR primers, 1X Amplitaq Gold buffer (15 mM Tris-HCl, pH 8.0, 50 mM KCl) and 1.25 units Amplitaq Gold DNA Polymerase (Thermo Fisher Scientific, 4311816) and 2 µl cDNA ("plus RT" or "no RT" depending on the tube).

The reaction mix was mixed, centrifuged at 3600g for 4 seconds and run in the PCR machine at the following settings; a first cycle of 95°C for 5 minutes (to activate the enzyme), 35 cycles of 95°C for 20 seconds, 42°C for 20 seconds and 60°C for 1 minute, and a last cycle of 60°C for 10 minutes after which the sample was cooled to 4°C. A molecular marker, 5 µl of *FullRanger* 100 bp DNA Ladder (Norgen, 11800 or 11300) or Quick-Load® 100 bp DNA Ladder (New England Biolabs, N0467S) and 5 µl of PCR products in 1 µl of 5x GelPilot DNA loading dye (Qiagen, 239901), were run by gel electrophoresis on a 1% agarose

gel (Thermo Fisher Scientific, 15510-027) with 1X TBE (1 M Tris, 0.9 M boric acid, and 0.01 M EDTA) buffer (Thermo Fisher Scientific, 15581-044) in dH_20 at 80 V for 45 to 60 minutes and stained with 0.5 µg/ml of ethidium bromide for 3 to 5 minutes. The PCR products were visualized under ultra violet light using the G:Box gel documentation system with GeneSnap software (Syngene).

4.4.3.4 Cloning of the DBLα tag: Purification and Ligation of the PCR product

The DBLα PCR product was purified using the Qiaquick PCR purification kit (Qiagen, 28104). Five volumes of Buffer PB, a high-salt buffer made up of 5 M guanidine hydrochloride and 30% isopropanol, was added to 1 volume of the PCR product. The resulting mix was added to a QIAquick spin column in a collection tube and centrifuged at 16,060g for one minute for adsorption of the DNA to the silica membrane in the column and the flow through discarded. The column was washed with 750 µl of Buffer PE (10 mM Tris-HCl pH 7.5 and 80% ethanol), centrifuged at 16,060g for one minute and the flow through discarded. The column was centrifuged again at 16,060g for one minute and the flow through discarded to remove residual Buffer PE. The column was placed in a fresh Eppendorf tube and 50 µl of Buffer EB, a low-salt elution buffer made of 10 mM Tris-Cl, pH 8.5, was added to the center of the column and left to stand for one minute after which the tube with the column was centrifuged at 16,060g for one minute to elute the bound DNA.

The purified PCR product was ligated into a PCR II vector (Thermo Fisher Scientific, K2070) with *Eco*R I sites for excision of inserts. For the ligation, 1 µl each of the PCR II vector, 10X ligase buffer (250 mM Tris-HCl pH 7.6, 50 mM $MgCl_2$, 5 mM ATP, 5 mM DTT and 25% w/v polyethylene glycol-8000) and T4 DNA ligase (Thermo Fisher Scientific, 15224-041) was added to 7 µl of the purified PCR product. The reaction mix was incubated overnight at 16°C and used immediately for transformation or stored at -20°C before use.

4.4.3.5 Transformation of *E. coli*

One Shot TOP10F´ chemically competent *E. coli* (Thermo Fisher Scientific, C3030), which has the *lac*I repressor for IPTG (Isopropyl β-D-1-thiogalactopyranoside) inducible expression from *lac*, *tac or trc* promoters was used for the transformation. The ligation reaction was centrifuged for 5 seconds and placed on ice. A 50 µl vial of OneShot TOP10F' was thawed on ice and 2 µl of the ligation reaction added and stirred gently. The bacteria suspension was incubated on ice for 30 minutes and heat-shocked by incubating at 42°C for 30 seconds after which it was quickly put on ice. Two hundred and fifty µl of Super Optimal broth with Catabolite repression (S.O.C medium) (Thermo Fisher Scientific, 15544-034; made up of 2% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, and 20 mM glucose) was added to the bacteria and the bacteria suspension grown at 37°C and 225 rpm for 1 hour.

Under aseptic conditions, a Luria Bertani (LB) agar plate was made with sterile 35 g/l LB-agar (Sigma-Aldrich, L2897) in dH_20 and 50 µg/ml ampicillin. When the plate had cooled and set, 40 µl each of 20 mg/ml X-gal and 100 mM IPTG were spread onto the plate and incubated at 37°C for 1 hour. After incubation, 100 µl of the transformed bacteria was evenly spread onto the prepared plate and incubated overnight at 37°C. The plate was used for minipreps or stored at 4°C until it was required for use.

4.4.3.6 Minipreps from transformed *E. coli*

At least 30 white colonies (cells transformed with the plasmid) were picked and each grown in 2 ml of sterile 20 g/l LB-Broth in dH_20 (Sigma-Aldrich, L3022) with 50 µg/ml ampicillin overnight at 225rpm and 37°C. The plasmids from the bacteria were extracted using QIAprep Spin Miniprep Kit, (Qiagen, 27106). For each of the minipreps, the bacteria culture was centrifuged at 12,350g for 3 minutes and the pellet resuspended in 250 µl of Buffer P1, a chaotropic salt of high concentration with RNase, which lysed the cells. The suspension was mixed thoroughly by inverting the tube 5 times till the solution was clear and 350 µl of

Buffer N3 (4.2 M GuHCl, 0.9 M KAc, pH 4.8) was added and the suspension mixed immediately by inverting the tube 6 times. The suspension was centrifuged at 16,060g for 10 minutes and the supernatant decanted into the Qiaprep spin column.

The column was centrifuged at 16,060g for 1 minute, for adsorption of the plasmid to the silica membrane, and the flow through discarded. The column with the plasmid was washed with 500 µl of Buffer PB, centrifuged at 16,060g for 1 minute and the flow through discarded. The wash was repeated with 750 µl of Buffer PE. The flow through was discarded and the column, centrifuged again at 16,060g for 1 minute to remove any residual buffer. The column was put into an Eppendorf tube and 50 µl of Buffer EB added to the center of the column and left to stand for 1 minute. The plasmid was eluted after centrifugation at 16,060g for 1 minute.

4.4.3.7 Restriction digest to check for inserts

A master mix was made with 0.75 µl of *Eco*R I (Promega, R6011), 0.5 µl of buffer H (Promega, R008A-C), and 3.75 µl of ddH_20. The volumes were scaled up depending on the number of samples. Five µl of the master mix was added to 5 µl of each of the plasmids and the reaction mix, incubated at 37°C for 2 hours. The samples were centrifuged at 3600g for 4 seconds and loaded together with a molecular marker on a 1% agarose gel and run in 1X TBE buffer in dH_20 at 80 V for 40 - 45 minutes. The gel was stained with 0.5 µg/ml of ethidium bromide for 3 to 5 minutes and the PCR products visualized under ultra violet light using the G:Box with GeneSnap software. Plasmids that had an insert at the expected molecular weight were taken further for sequencing.

4.4.3.8 Sequencing of plasmids

A BigDye (Thermo Fisher Scientific, 4337457) reaction was carried out for the plasmids that had inserts of the expected size (300-500bp). This involved the

addition of 1 μl of 1.6 μM VF1 primers (AGATGCATGCTCGAG CGG) and 2 μl each of BigDye and ddH20 to 5μl of each of the plasmids. The samples were run on the PCR machine with the following settings; 96°C for 30 seconds and 50°C for 15 seconds for 25 cycles and 60°C for 4 minutes. The PCR products were sent to Edinburgh Genomics, University of Edinburgh for sequencing (http://genomics.ed.ac.uk/).

4.4.3.9 Analysis of sequences

The quality of the sequences was determined by checking the base calls of the nucleotide sequence chromatograms on DNAStar. The sequences were translated to the protein sequence using the ExPASY translation tool (http://web.expasy.org/translate/) and those without the sequences 'ARSFADIG' at the 5' and 'PQYLRW' at the 3' end were excluded. The sequences obtained were used to classify the PfEMP1 based on the number of cysteine residues; where two cysteine residues in the DBLα sequence meant it was a group A or B/A PfEMP1, and four cysteine residues meant it was a group B or C PfEMP1 (Bull *et al.*, 2005). All sequences will be deposited in the GenBank database.

All the sequences were aligned by Clustal W in MegAlign 13 (DNAStar) and sequences having >96% identity, were considered as the same. A percentage of each of the different DBLα sequence in the total DBLα sequences was calculated. For 9197 IEs whose genome had been sequenced, the DBLα tag sequences were used to determine the full *var* genes transcribed by the 9197 unselected and HBEC selected 9197 in an alignment of the translated protein sequences using Clustal Omega in MegAlign Pro 13 (DNAStar). 9197 *var* gene sequences were obtained from Dr. Peter C. Bull, University of Cambridge.

To classify the 9197 PfEMP1s into the groups A, B, C and the strain transcendent *var1*, *var2csa* (group E) and *var3* types, the 9197 *var* gene sequences were aligned with at least 5 sequences for each PfEMP1 group or type from the HB3, IT4 (FCR3) and 3D7 parasite lines by Clustal Omega in

MegAlign Pro 13. The PfEMP1 with the known groups were randomly selected from Rask et al. (Rask *et al.*, 2010) and included all the twenty reported DC8 and DC13 PfEMP1 which were from the HB3, IT4 (FCR3), PFCLIN, RAJ116, IGH, DD2 and MC parasite lines. The sequences of all the parasite lines were obtained from the VarDom 1.0 Server (http://www.cbs.dtu.dk/services/VarDom/) (Lavstsen *et al.*, 2012). The phylogenetic trees obtained from the alignments were all exported to Figtree to create a polar or radial tree layout.

4.4.3.10 Determination of PfEMP1 domains of 9197 and 8211 IEs

Using the VarDom 1.0 Server, the NTS, DBL and CIDR domains of twenty DC8 and DC13 PfEMP1 (From HB3, IT4, 3D7, PFCLIN, RAJ116, IGH, DD2, MC IEs) and seven group A PfEMP1 (DD2var09a, DD2var40, IGHvar26, IGHvar32 PFE1640w, PF08_0141, PFCLINvar48) were selected to obtain a wide range of the different types of group A or B/A domains. The NTS, DBL or CIDR domains of the 9197 and 8211 PfEMP1 sequences of interest, were aligned with the corresponding domains of known PfEMP1 by Clustal Omega in MegAlign Pro 13. The phylogenetic tree generated from the alignment was exported to Figtree to create a polar or radial tree layout, and the 9197 or 8211 domains and the known domains they clustered with were represented by the same colours on the tree.

4.4.4 Static binding inhibition assays with PfEMP1 domain proteins and antibodies

Binding assays were carried out in 48 well plates as described in sections 2.4 and 2.5, with a 20-minute pre-incubation step. For the protein inhibition, HBEC-5i were pre-incubated with 190 µl of 50 µg/ml of the following recombinant proteins in 0.1% BSA/DMEM binding medium pH 7.3; NTS.DBLα, DBLβ and CIDRα1 of HB3var03, IT4var07 and IT4var19, NTS.DBLα-CIDRα1 of HB3var03, and CIDRα1-DBLβ didomain of HB3var03 and IT4var19. The NTS.DBLα of

TM284, and CIDRα1 and DBLβ of SAO75 (both TM284 and SAO75 were non-HBEC binding strains) were used as negative controls. The HBEC-5i were incubated with the recombinant proteins for 20 minutes with a resuspension by swirling after 10 minutes of incubation. Gelatin purified trophozoites at not less than 30% parasitaemia were added to the HBEC-5i to make up 2% haematocrit in each well and incubated for one hour at 37°C.

For antibody inhibition, gelatin purified trophozoites (≥ 30% parasitaemia) were incubated at 2% haematocrit with 50 µg/ml of antibodies to the proteins above raised in rabbit or rabbit IgG (isotype control), in 0.1% BSA/DMEM binding medium, pH 7.3. The IEs-antibody suspension was pre-incubated for 20 minutes with a resuspension by flicking the tubes after 10 minutes. After the incubation, 190 µl of the suspension was added to the HBEC-5i in each well and the cells were co-incubated for one hour at 37°C. The cells were washed, fixed and the number of IEs bound counted blinded to the identity of the samples. Binding was compared with control wells that contained IEs and HBEC-5i in 0.1% BSA/DMEM binding medium only.

4.5 Results

4.5.1 Selection of SAO75 IEs for binding to HBEC-5i

SAO75 IEs were panned on HBEC-5i (referred to as SA075.HBEC after selection on HBEC-5i) but showed was no significant increase in adhesion to HBEC-5i ($p = 0.23$) after ten rounds of selection (Figure 4.2). IEs have been shown to bind to endothelial cells by knobs on the surface of the cells (Udeinya *et al.*, 1981) therefore the lack of binding after several rounds of panning may have been due to absence of knobs (Claessens *et al.*, 2012) on the surface of the IEs.

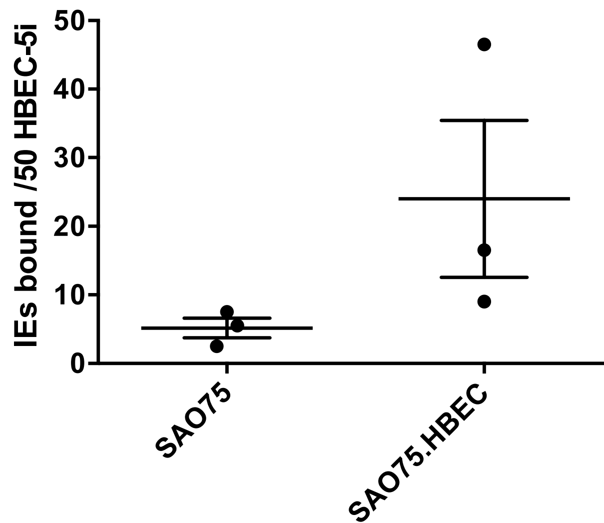


Figure 4.2. SAO75 IEs show no significant increase in cytoadhesion after HBEC-5i selection. Pigmented trophozoites (5-10% parasitaemia) of the unselected SAO75 and the SAO75.HBEC that had been panned ten times on HBEC-5i, were made up to 2% haematocrit in 0.1% BSA/DMEM binding medium and co-incubated with HBEC-5i at 37°C for 75 minutes. Data shown are the mean and SEM of three experiments (n = 3), each done with triplicate wells. Using a paired t-test, there was no significant difference between adhesion of SAO75 and SAO75.HBEC to HBEC-5i.

SAO75 and the other two parasite lines 8211 and 9197 were gelatin purified for the detection of knobs on the surface of the IEs. SAO75 IEs did not have knobs as no KAHRP was detected on their permeabilised surface membranes (Figure 4.3). More than half of the 9197 (62%) and 15% of 8211 IEs expressed KAHRP. The positive control HB3 brightly stained for KAHRP (75%), while no staining was seen for KAHRP on DD2, a knob-negative strain (Figure 4.3).

SA075 was cloned twice during its adaptation to culture. It was possible the 'knobless' form was generated during one of these cloning steps. I therefore examined the pre-clone SA075 by staining the IEs for KAHRP and found 78% were positively stained for KAHRP. (Figure 4.3).

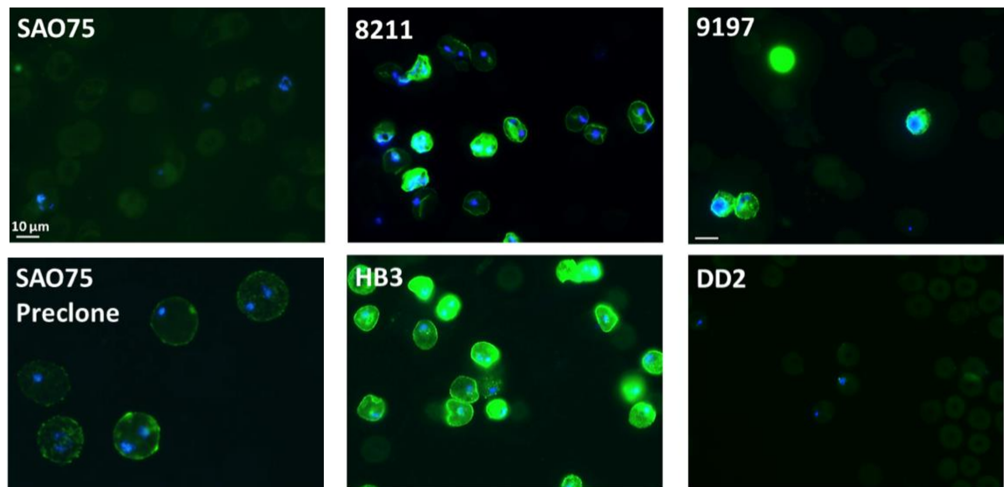


Figure 4.3. Immunofluorescence staining for knobs on the surface of SAO75, 9197, 8211 and SAO75 (pre-clone) IEs, with HB3 and DD2 IEs as controls. Gelatin purified pigmented trophozoites were incubated with 1/1000 KAHRP mAb 89, washed and the secondary incubation done with 1/1000 Alexa Fluor 488 goat anti-mouse IgG in DAPI. The cells were washed and mounted for fluorescent detection. The green (Alexa Fluor 488) fluorescence is positive staining for KAHRP and the blue fluorescence is DAPI stain of the parasite nuclei.

The pre-clone SAO75 was panned on HBEC-5i four times and both the selected and unselected bound to HBEC-5i with no difference in binding (Figure 4.4). The SAO75 IEs were found to bind to recombinant CSA (Figure 4.5; method in section 2.6), a receptor expressed on HBEC-5i. Selection of SAO75 was not pursued any further as the absence of differences between the unselected and the HBEC-selected SAO75 (SAO75-HBEC Pre-clone) suggested the PfEMP1 expression profile of these IEs might not be specific to HBEC-binding.

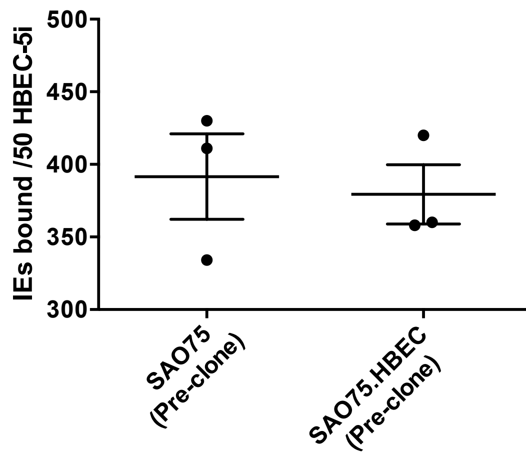


Figure 4.4. Adhesion of SAO75 (pre-clone) and SAO75.HBEC (pre-clone) to HBEC-5i. Pigmented trophozoites (5-10% parasitaemia) of the unselected SAO75 or the SAO75.HBEC that had been panned four times on HBEC-5i, were made up to 2% haematocrit in 0.1% BSA/DMEM binding medium and co-incubated with HBEC-5i at 37°C for 75 minutes. Data shown are the mean and SEM of three experiments, n = 3, each done with triplicate wells. No significant difference in binding (p = 0.23) was observed when compared in a paired t-test.

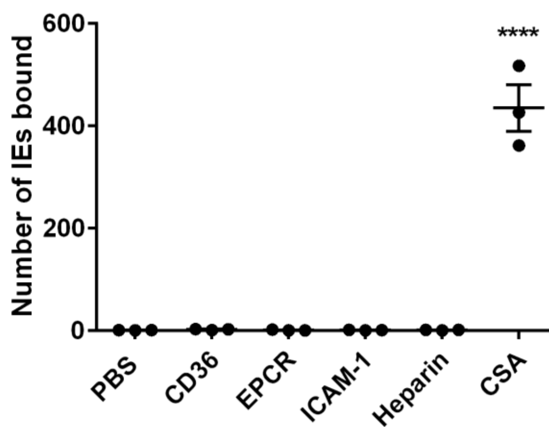


Figure 4.5. SA075.HBEC (pre-clone) IEs binds to CSA. IEs of at least 10% parasitaemia in 0.1% BSA/DMEM binding medium were incubated with 50 µg/ml of receptor molecules absorbed on a dish for an hour at 37°C. Each experiment included duplicate dishes, each with two spots per receptor and IEs were counted for three fields per spot at 400X. The data shown are mean and SEM of three independent experiments. Comparisons to the PBS control were made using a repeated measures one-way ANOVA, n = 3; **** P <0.0001.

4.5.2 Selection of 9197 and 8211 for binding to HBEC-5i

9197 and 8211 IEs were gelatin purified and selected for binding to HBEC-5i. After 5 rounds of panning, 9197 IEs (referred to as 9197.HBEC after selection on HBEC-5i), showed a >20-fold increase in binding to HBEC-5i compared to the unselected 9197 (Figure 4.6). 8211 IEs also selected for HBEC-5i binding (here after referred to as 8211.HBEC) also bound to HBEC-5i about 5 fold more than the unselected after 5 rounds of panning.

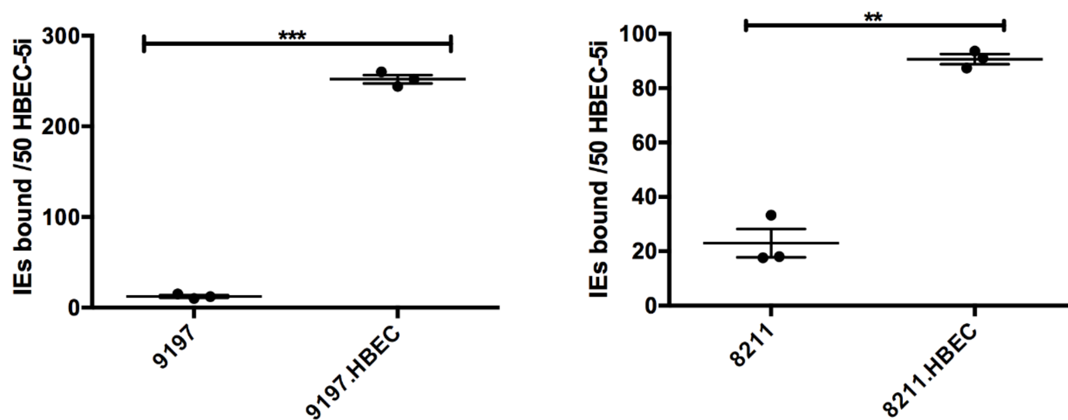


Figure 4.6. Increase in cytoadhesion of 9197 and 8211 after selection for HBEC-binding. The binding assay was done after 5 rounds of panning on HBEC-5i. Pigmented trophozoites (5-10% parasitaemia) in DMEM binding medium/0.1% BSA were co-incubated with HBEC-5i at 37°C for 75 minutes. Data shown are the mean and SEM of three experiments (n = 3) each done with triplicate wells. Comparisons were made using a paired t-test; ** $P < 0.01$, *** $P < 0.0001$.

4.5.3 *Var* gene expression after selection for binding to HBEC-5i

After at least 5 rounds of panning on HBEC-5i (5 and 8 rounds for 8211 and 9197 respectively), mRNA was extracted from both selected and unselected parasites and the DBLα transcripts amplified by rt-PCR with the degenerate DBLα primers (Bull *et al.*, 2005). DBLα transcripts of approximately 400 – 500bp (Figure 4.7) were obtained for ligation into plasmids and subsequent

transformation of *E. coli.* At least 30 recombinant plasmids with the inserts (Figure 4.8) were sequenced for the unselected and selected IEs.

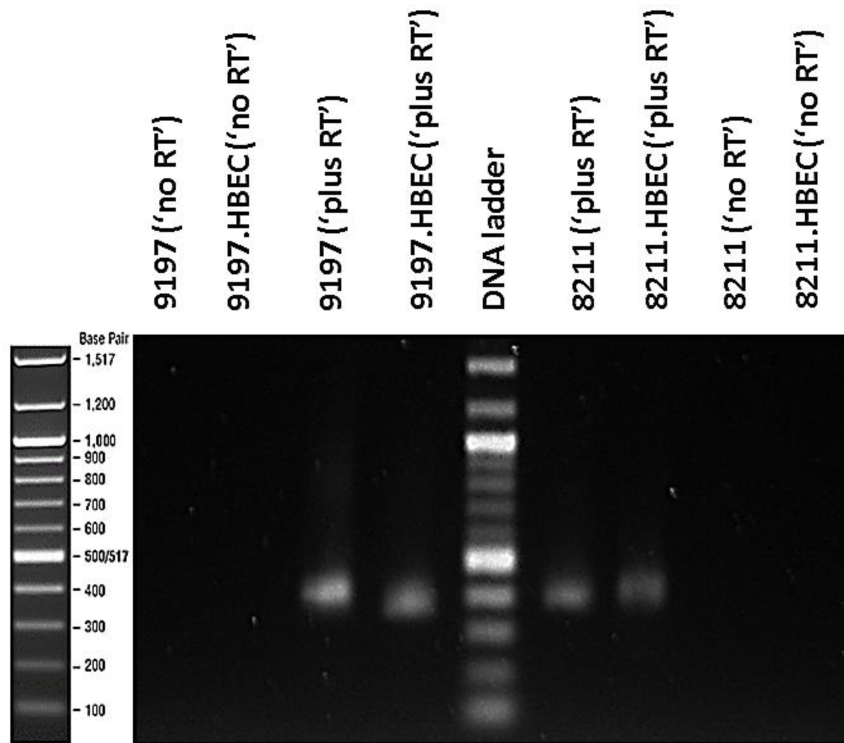


Figure 4.7. DBLα RT-PCR products of 9197, 9197.HBEC, 8211 and 8211.HBEC. The cDNA of the unselected and selected 9197 and 8211 were amplified with the DBLα αAF' and αBR primers and the products were run by gel electrophoresis on 1% agarose gel at 80 V for 45 minutes. The samples that had the reverse transcriptase (RT) are labelled as 'plus RT' and the controls that had dH_20 instead of the RT enzyme, to ensure there was no contamination from the genomic DNA, are labelled as 'no RT'.

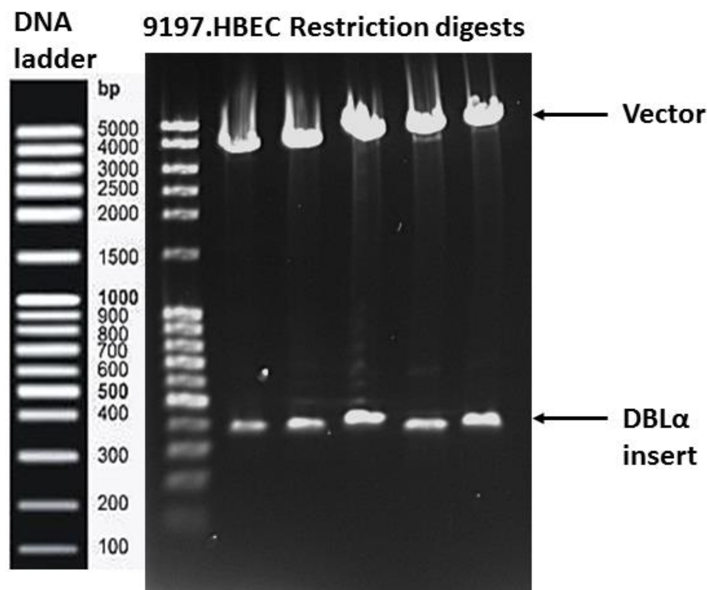


Figure 4.8. A representative gel of miniprep clone digests. The plasmids obtained from the *E. coli* colonies after transformation, were digested with *Eco*R I for 2 hours at 37°C and the products run on 1% agarose gel at 80 V for 45 minutes to determine the plasmids that had the insert of approximately 300 to 500bp.

4.5.3.1 9197

Thirty-two and thirty-nine recombinant plasmids with the insert were sequenced for the unselected 9197 and selected, 9197.HBEC respectively. For each, 25 readable sequences were obtained for 9197 and 26 for 9197.HBEC. With the selected 9197.HBEC, the *var* genes predominantly transcribed were identified as *9197var15* and 9197*var*27 (Figure 4.9).

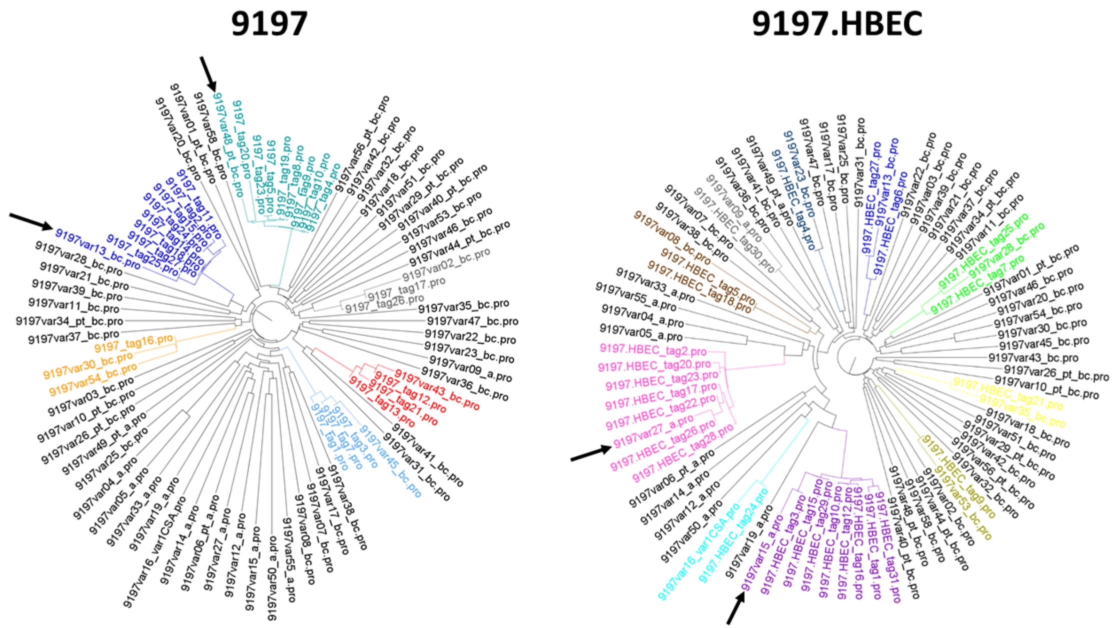


Figure 4.9. Phylogenetic trees of DBLα tags of the 9197 and 9197.HBEC with 9197 PfEMP1 (s). The DBLα tag nucleotide sequences obtained from the unselected (9197) and the selected 9197 (9197.HBEC) were translated to proteins and each was aligned with full length 9197 PfEMP1 sequences (obtained from Dr. Peter C. Bull) using Clustal Omega in MegAlign Pro. The phylogenetic trees generated, were exported to Figtree to create a radial tree layout. The full length 9197 PfEMP1 are labelled as 'name of PfEMP1_PfEMP1 group predicted from the number of cysteines.pro'. Labels with 'pt' are partial sequences of the PfEMP1. The DBLα tags (sequences of >96% identity) and the full length 9197 PfEMP1 they align with are shown in the same colour. The arrows show the PfEMP1 that were predominantly transcribed. The rest of the 9197 full length PfEMP1 sequences that were not detected, as expressed DBLα tags, are in black.

The *9197var15* and *9197var27* formed 30.8% (8/26) and 26.9% (7/26) respectively of the *var* genes transcribed in the 9197.HBEC (Figure 4.10). These sequences were absent in the unselected 9197 which predominantly transcribed 9197*var*13 and 9197 *var*48; 32% (8/25) each.

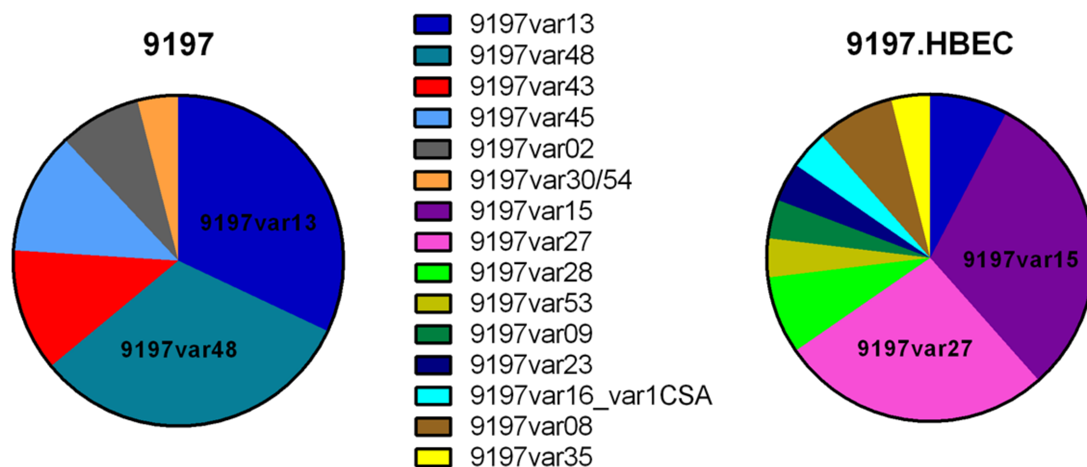


Figure 4.10. *Var* gene expression profile of 9197 and 9197.HBEC. The frequency of the DBLα tags, obtained after RT-PCR with universal PfEMP1 DBLα degenerate primers for the unselected IEs and HBEC-selected IEs, is represented in the pie chart. A total of 25 protein sequences for 9197, and 26 protein sequences for 9197.HBEC were obtained.

The genome of 9197 was recently sequenced and the *var* genes had not been fully annotated and classified. PfEMP1 from the 58 *var* genes present in the genome were aligned with all DC8 and DC13 PfEMP1 (Rask *et al.*, 2010) and at least 5 PfEMP1 from each of the groups, A, B, C, E, var1 and var3 for 3D7, HB3 and IT4 parasites (Figure 4.11). The alignment generated two phylogenetic trees with groups A (including var1 and var3) on one tree and groups B, C and E on the other. There was one sequence 9197var48 that did not form part of the trees. This may be because it was a partial sequence. With the exception of five 9197 PfEMP1 (var 4, 5, 9, 33 and 49), which had 2 cysteine residues but aligned with the groups B and C, classification of the PfEMP1 into group A, B or C, based on the number of cysteine residues match the groups identified after alignment.

The *var* genes predominantly transcribed in the IEs that bound to HBEC-5i, 9197*var*15 and 9197*var*27 were group A-like *var* genes (Figure 4.11). The 9197var15 PfEMP1 aligned with group A PfEMP1 encoded by upsA *var* genes and 9197var27 with groups B/A and DC8 PfEMP1. The 9197var13, whose encoding gene decreased after selection on HBEC-5i (Figure 4.10), aligned with group C PfEMP1 (Figure 4.11) while 9197var48 (*9197var48* was predominantly

transcribed in the unselected 9197) did not align with any of the known groups in Figure 4.11.

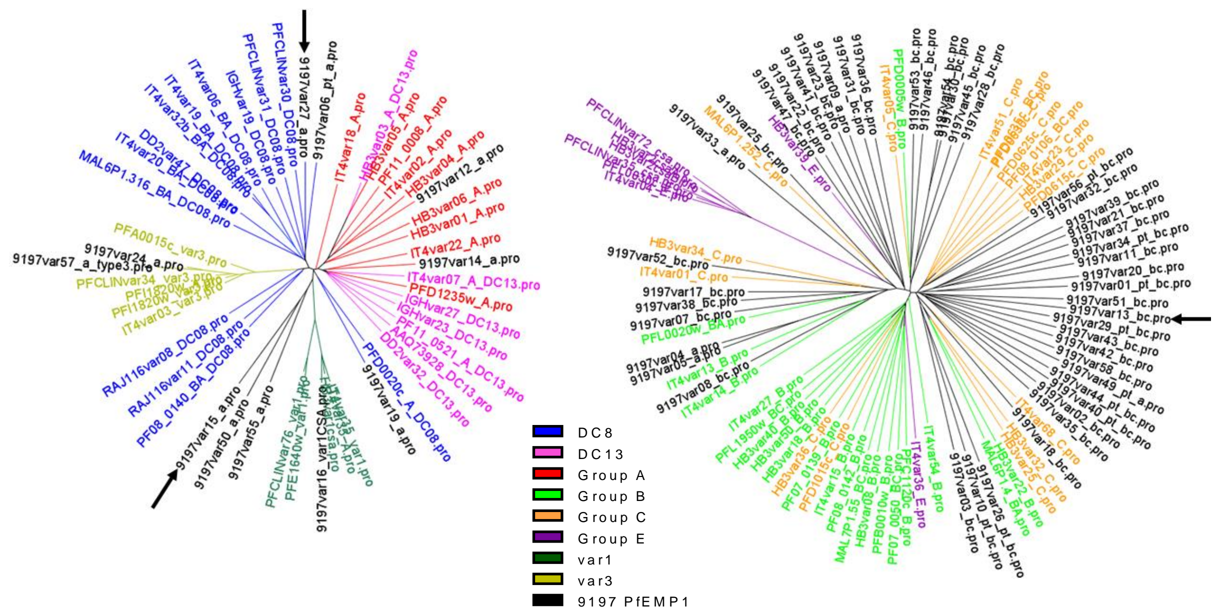


Figure 4.11. Phylogenetic trees of all 9197 PfEMP1 variants with known groups of PfEMP1 (A, B, C, E, var1 and var3) including DC8 and DC13 PfEMP1. At least five randomly selected *var* gene nucleotide sequences from groups A, B, C, E, *var1* and *var3* sequences of HB3, IT4 and 3D7 including twenty DC8 and DC13 sequences (From HB3, IT4, 3D7, PFCLIN, RAJ116, IGH, DD2, MC) were translated to the PfEMP1 proteins. All 9197 PfEMP1 variants and the known sequences were aligned with Clustal Omega in MegAlign Pro. Each of the known PfEMP1 sequence is labelled as 'name of PfEMP1_group of PfEMP1 and or domain cassette.pro'. The arrows show the 9197 PfEMP1 (s) that were predominantly transcribed by the 9197.HBEC IEs (9197var15 and 9197var27) and the unselected 9197 IEs (9197var13). 9197var48 predominantly transcribed by 9197 IEs did not form part of the trees.

Based on the domain classes obtained from the VarDom 1.0 server and an alignment of the 27 group A-like (13 DC8, 7 DC13 and 7 other group A) PfEMP1 (Figure 4.12), the domain architecture of 9197var15 and 9197var27 was predicted to be as shown in Figure 4.13. The 9197var27 has the NTSB and DBLα2 of group B and the DBLα2-CIDRα1.1-DBLβ12-DBLγ4 domains of DC8 PfEMP1 while the 9197var15 had the NTSA and DBLα1 of a group A PfEMP1.

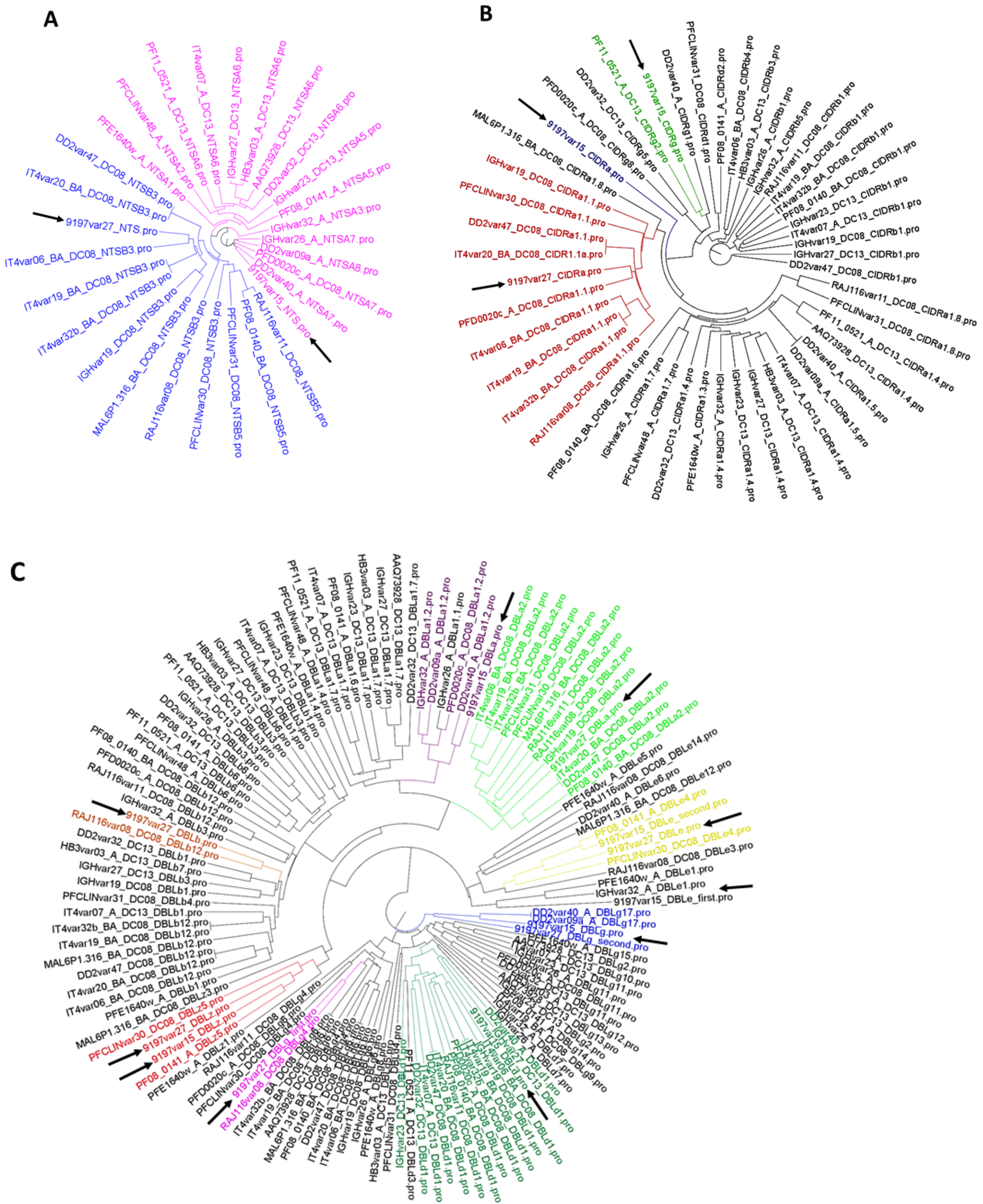


Figure 4.12. Phylogenetic trees of NTS, DBL and CIDR domains of 9197var15 and 9197var27, and known group A or B/A PfEMP1 including DC8 and DC13 PfEMP1. The NTS (A), CIDR (B) and DBL (C) domains of PfEMP1 sequences of 9197var15, 9197var27, seven group A PfEMP1 and twenty DC8 and DC13 PfEMP1 (from HB3, IT4, 3D7, PFCLIN, RAJ116, IGH, DD2, MC) were determined from the domain block prediction from the VarDom 1.0 Server. The protein

sequences were aligned with Clustal Omega in MegAlign Pro and the phylogenetic trees generated were exported to Figtree to create a radial tree layout. Each protein sequence is labelled as 'name of PfEMP1_group of PfEMP1 and or domain cassette_domain.pro'. Same colours show 9197 PfEMP1 domains and the PfEMP1 domains they cluster with. The 9197 PfEMP1 domains are indicated by arrows.

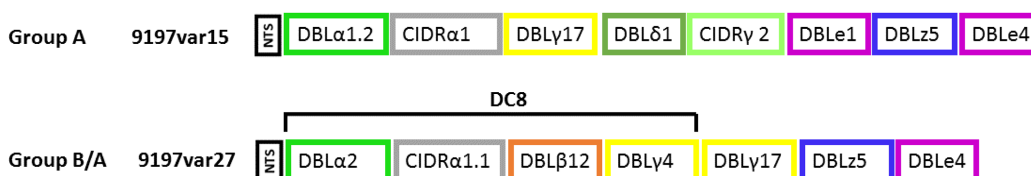


Figure 4.13. Predicted PfEMP1 domain architecture of 9197var15 and 9197var27. 9197var15 has the N-terminal DBLα1 domain of group A PfEMP1 and 9197var27 has the tandem arrangement of domains characteristic of DC8 and group B /A PfEMP1.

4.5.3.2 8211

At least 30 recombinant plasmids each were sequenced; and 25 readable sequences were obtained for 8211 and 28 were obtained for 8211.HBEC. The genome of 8211 has not been sequenced yet; therefore, the contigs have been labelled as DBLα_tags. The DBLα_tag1 was upregulated after selection on HBEC-5i with an increase from 4% to 32.1% (Figure 4.14). There were two others, DBLα_tag18 and DBLα_tag19 that were also predominantly expressed (28.6% and 25% respectively) after selection on HBEC-5i. The DBLα_tag4 and DBLα_tag5, which were abundant before selection, were absent in the 8211.HBEC.

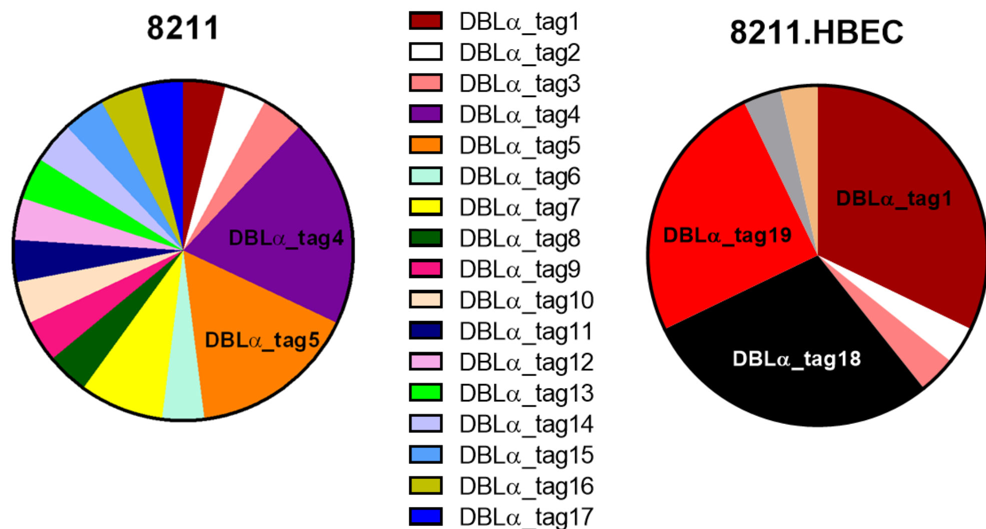


Figure 4.14. *Var* gene expression profile of 8211 and 8211.HBEC. The frequency of the DBLα tags, obtained after RT-PCR with universal PfEMP1 DBLα degenerate primers for the unselected IEs and HBEC-selected IEs, is represented in the pie chart. A total of 25 protein sequences for 8211 and 28 protein sequences for 8211.HBEC were examined.

Based on the number of cysteine residues and alignment with known groups of full length PfEMP1 in Figure 4.15, the DBLα_tag1, DBLα_tag18 and DBLα_tag19 were classified as group A-like PfEMP1 while the unselected 8211 expressed mostly group B or C. The DBLα_tag1 clustered with DC8 PfEMP1 (Figure 4.15) and was identified to be a DBLα2 domain (Figure 4.16). The DBLα_tag18 and DBLα_tag19 could only be identified as group A but they were both DBLα1.2 domains, similar to that seen in the DC8-like HBEC-binding PFD0020c of 3D7 (Claessens *et al.*, 2012).

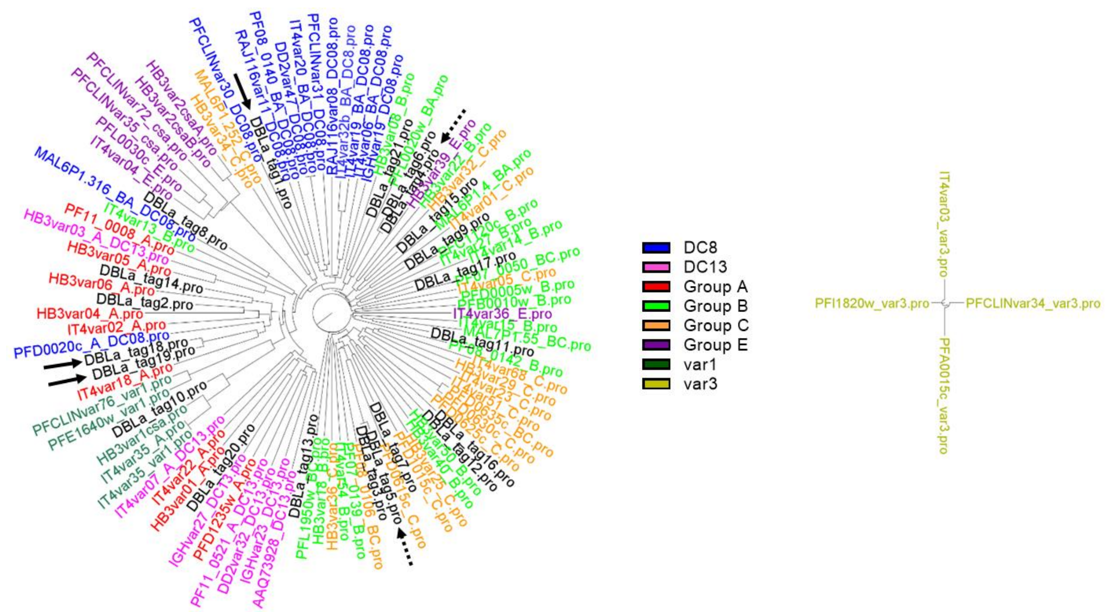


Figure 4.15. Phylogenetic trees of 8211 DBLα tags with full length DC8, DC13 and other known groups of PfEMP1. At least five randomly selected *var* gene nucleotide sequences from groups A, B, C, E, *var1* and *var3* sequences of HB3, IT4 and 3D7 including twenty DC8 and DC13 sequences (From HB3, IT4, 3D7, PFCLIN, RAJ116, IGH, DD2, MC) were translated to the PfEMP1 proteins. The protein sequences of 8211 DBLα tags that were predominantly transcribed by the unselected and selected 8211 were aligned with Clustal Omega in MegAlign Pro. The alignment generated two phylogenetic trees that were exported to Figtree to create a radial tree layout. Each protein sequence is labelled as 'name of PfEMP1_group of PfEMP1 and or domain cassette.pro' and the 8211 sequences, which are labelled as DBLa_tags are shown by arrows.

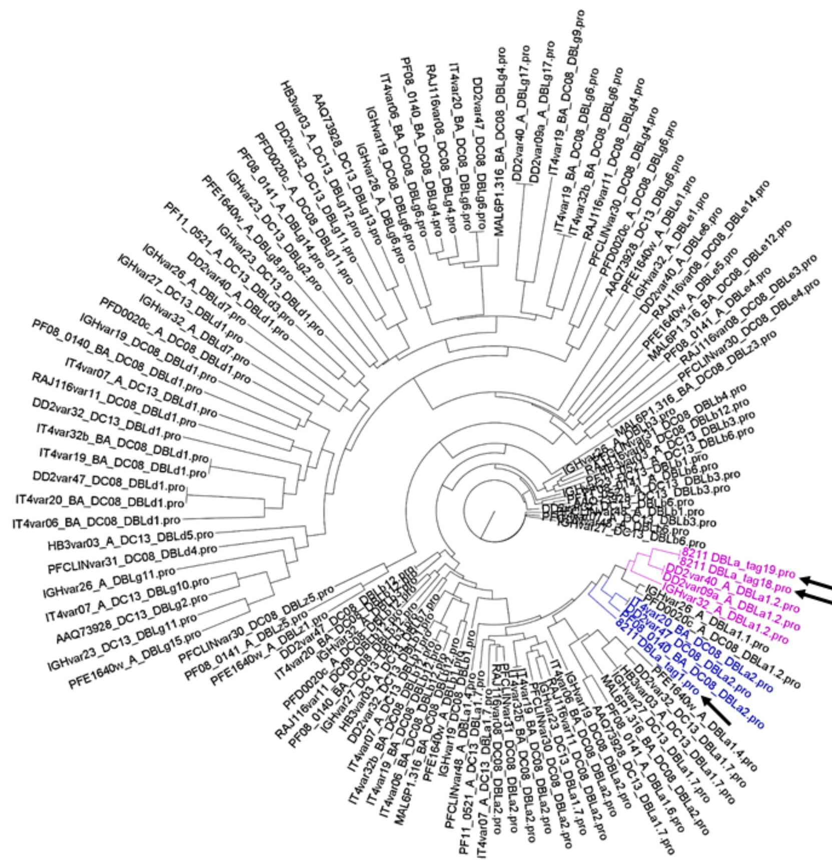


Figure 4.16. Phylogenetic tree of 8211 DBLα tags and DBL domains of known group A or B/A PfEMP1 including DC8 and DC13 variants. The 8211 DBLα tags 1, 18 and 19 predominantly transcribed by 8211.HBEC, and DBL domains of seven group A PfEMP1 and twenty DC8 and DC13 PfEMP1 (From HB3, IT4, 3D7, PFCLIN, RAJ116, IGH, DD2, MC) were aligned with Clustal Omega in MegAlign Pro and the phylogenetic trees generated were exported to Figtree to create a radial tree layout. Each protein sequence was labelled as 'name of PfEMP1_group of PfEMP1 and or domain cassette_domain.pro' and the 8211 domains are labelled as DBLa_tags. 8211 DBLα tags and the PfEMP1 domains they cluster with, are shown by the same colours with the 8211 DBLα tags are indicated by arrows.

4.5.4 The role of PfEMP1 domains in mediating cytoadhesion to HBEC

4.5.4.1 Effect of PfEMP1 domain recombinant proteins

For the identification of the domain involved in adhesion to HBEC, the NTS.DBLα, CIDRα1 and DBLβ of HB3var03, IT4var07 and IT4var19 and the

didomains, NTS.DBLα-CIDRα of HB3var03, and CIDRα-DBLβ of HB3var03 and IT4var19, were expressed as recombinant proteins in *E. coli* by Dr. Gabriella Lindergard and Dr. Ashfaq Ghumra, and used in static HBEC binding inhibition assays. The proteins were at the expected molecular weight and showed a shift upon reduction indicating disulphide bonds in the protein.

Although the yield of the NTS.DBLα recombinant proteins at the expected molecular weight was good, the proteins of three PfEMP1 variants lines had aggregates and fragments that compromised the quality of the proteins (Figure 4.17).

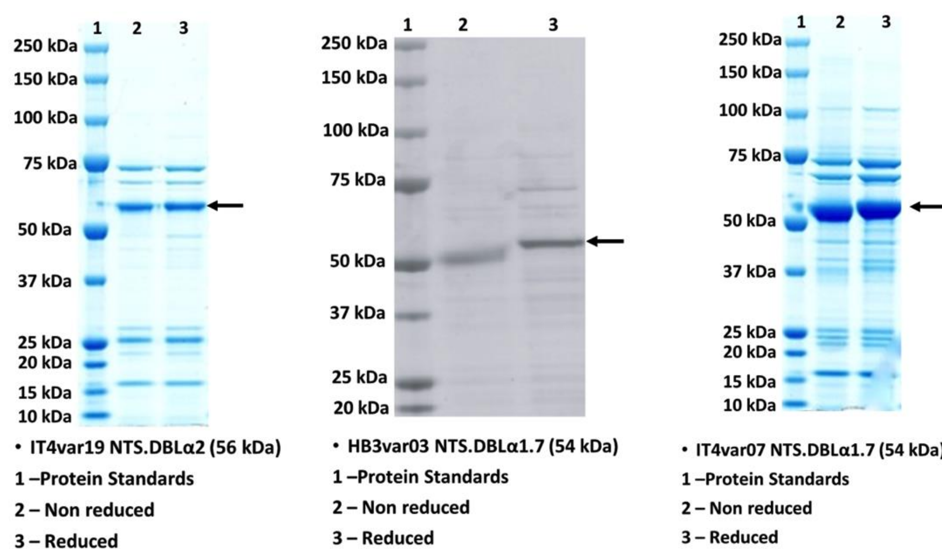


Figure 4.17. SDS-PAGE of recombinant NTS.DBLα1 domain proteins of IT4var19, HB3var03 and IT4var07 expressed in *E. coli*. A gel electrophoresis of 2 µg each of the reduced and non-reduced pairs of the proteins was run on 10% SDS-polyacrylamide proteins.

Adhesion of IT4var19, HB3var03 and IT4var07 IEs to HBEC-5i was not affected by the NTS.DBLα recombinant proteins (

Figure **4.18**). Due to the other bands in the SDS-PAGE, this result cannot be attributed to a specific effect of the NTS.DBLα recombinant proteins, as possible interactions of the fragments and aggregates with the NTS.DBL protein cannot be ruled out.

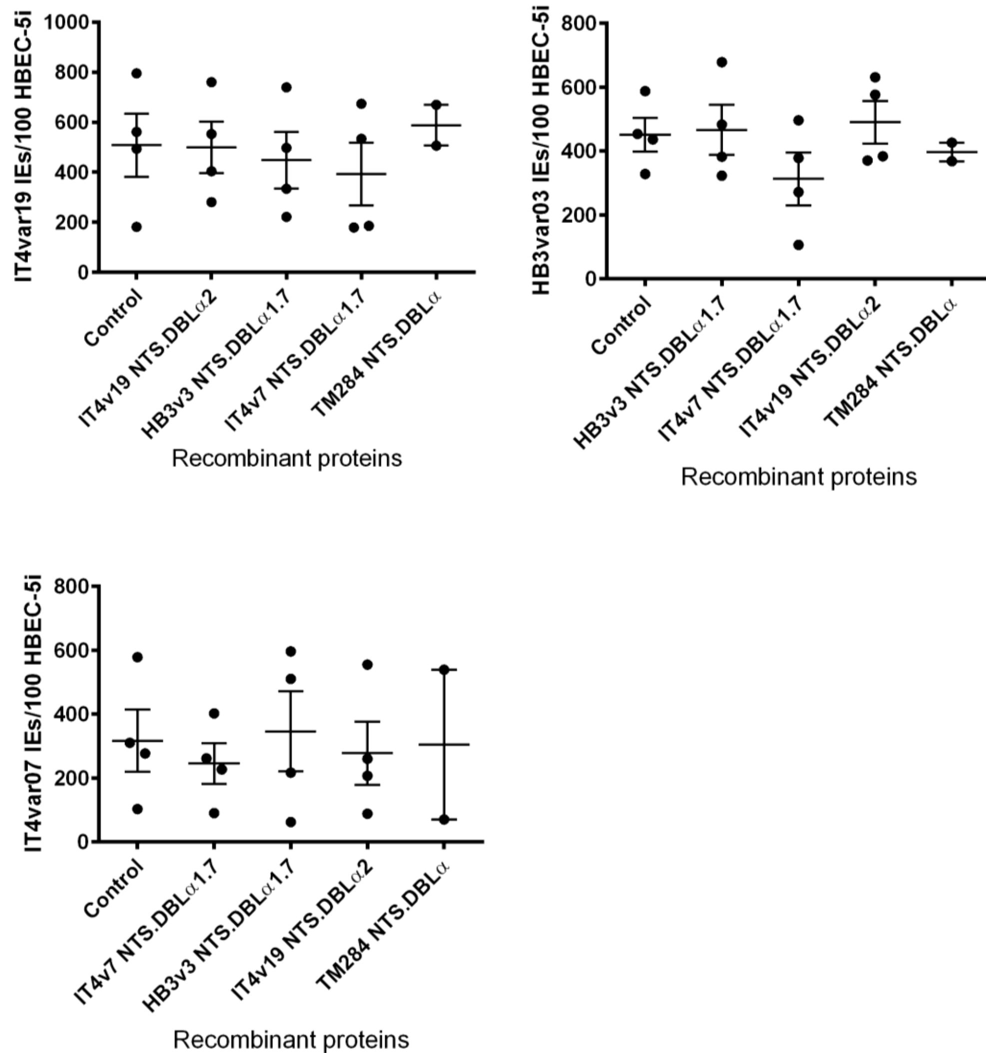


Figure 4.18. Effect of NTS.DBLα recombinant proteins on cytoadhesion of IT4var19, HB3var03 and IT4var07 IEs to HBEC-5i. HBEC-5i were pre-incubated with 50 μg/ml of the NTS.DBLα recombinant proteins in 0.1% BSA/DMEM binding medium before co-incubation with gelatin purified IEs (parasitaemia of at least 30%) for binding. Data shown are the mean and SEM of at least two experiments, each with duplicate wells. The log transformed values of the IEs bound were compared to that of the controls in a one-way ANOVA with Dunnett's multiple comparisons test ($n \geq 2$) and showed significant difference.

The HB3var03 and IT4var07 CIDRα1 recombinant proteins were of good quality, whereas the IT4var19 protein contained some fragments and aggregates (Figure 4.19).

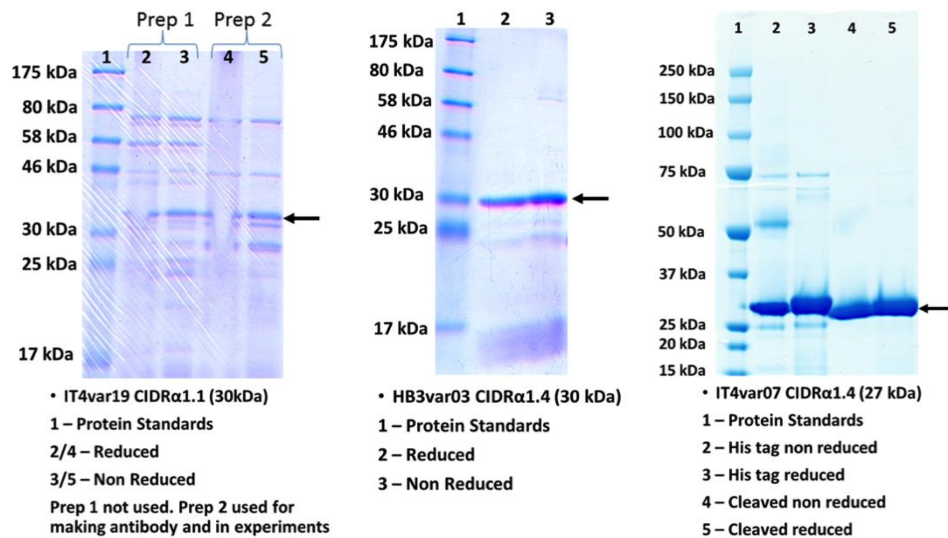


Figure 4.19. SDS-PAGE showing recombinant CIDRα1 domain proteins of IT4var19, HB3var03 and IT4var07 expressed in *E. coli*. A gel electrophoresis of 2 µg each of the reduced and non-reduced pairs of the proteins was run on 10% SDS-polyacrylamide proteins. IT4var07 CIDRα1 proteins before ('His tag') and after ('cleaved') cleavage of the His tag with TEV protease are also shown.

The three CIDRα1 recombinant proteins significantly inhibited adhesion of the DC8-expressing variant, IT4var19.IEs, to HBEC-5i but did not affect cytoadhesion of the DC13-expressing HB3var03 and IT4var07 IEs to HBEC-5i (Figure 4.20). EPCR has been shown to mediate binding of IT4var19 IEs to HBEC (Turner *et al.*, 2013) and the binding site of CIDRα1.1 and 1.4 to EPCR have been shown (Lau *et al.*, 2015). The result in Figure 4.20 suggests that CIDRα1.1 (the IT4var19 variant) and 1.4 (HB3var03 and IT4var07 variants) bind to HBEC and may have bound to EPCR as this prevented binding of IT4var19 IEs to HBEC-5i. The data also suggest that the CIDRα1 is essential for adhesion of IT4var19 IEs to HBEC-5i but may not be involved in cytoadhesion of HB3var03 and IT4var07 IEs to HBEC-5i.

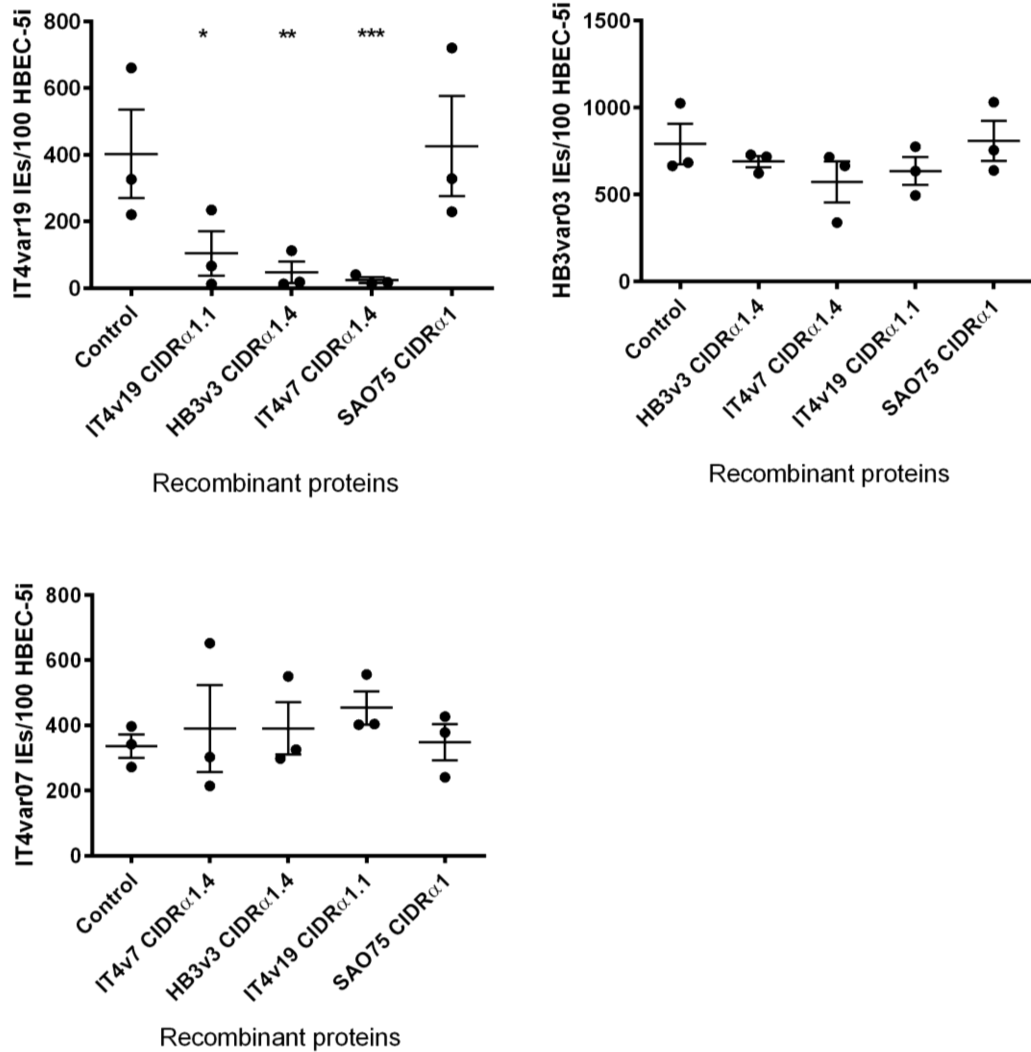


Figure 4.20. CIDRα 1.1 and 1.4 recombinant proteins inhibit cytoadhesion of only IT4var19 to HBEC-5i. HBEC-5i were pre-incubated with 50 μg/ml of the CIDRα1 recombinant proteins in 0.1% BSA/DMEM binding medium before co-incubation with gelatin purified IEs (parasitaemia of at least 30%) for binding. Data shown are the mean and SEM of three experiments, each with duplicate wells. The log transformed values of the number of IEs bound were compared to that of the controls using repeated measures one-way ANOVA with Dunnett's multiple comparisons test (n = 3). *P < 0.05. ** P < 0.01. *** P < 0.001.

The DBLβ recombinant proteins of IT4var19 and HB3var03 showed a prominent band at the expected molecular weight on the SDS gel in Figure 4.21.

The IT4var07 DBLβ recombinant protein, however, was not of good quality as it contained fragments and aggregates.

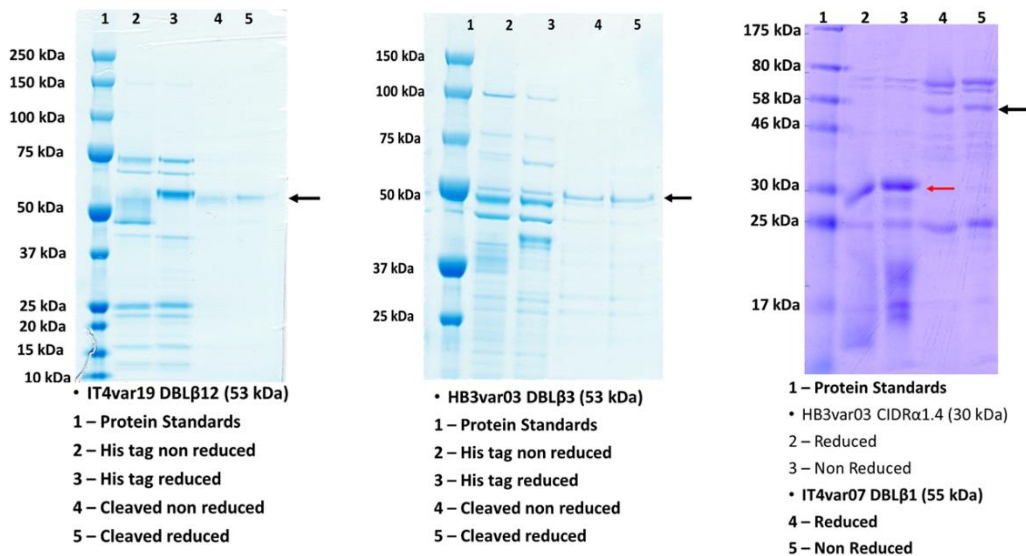


Figure 4.21. SDS-PAGE of recombinant DBLβ domain proteins of IT4var19, HB3var03 and IT4var07 expressed in *E. coli*. A gel electrophoresis of 2 μg each of the reduced and non-reduced pairs of the proteins was run on 10% SDS-polyacrylamide proteins. With the exception of IT4var07 DBLβ, the proteins are shown before ('His tag') and after ('cleaved') cleavage of the 'His tag' with TEV protease.

With the exception of the DBLβ1 domain of IT4var07, which inhibited the adhesion of HB3var03 IEs, the DBLβ recombinant proteins did not affect cytoadhesion of IT4var19, HB3var03 or IT4var07 IEs to HBEC-5i (Figure 4.22) suggesting that DBLβ domain may not mediate adhesion of all the three parasite lines.

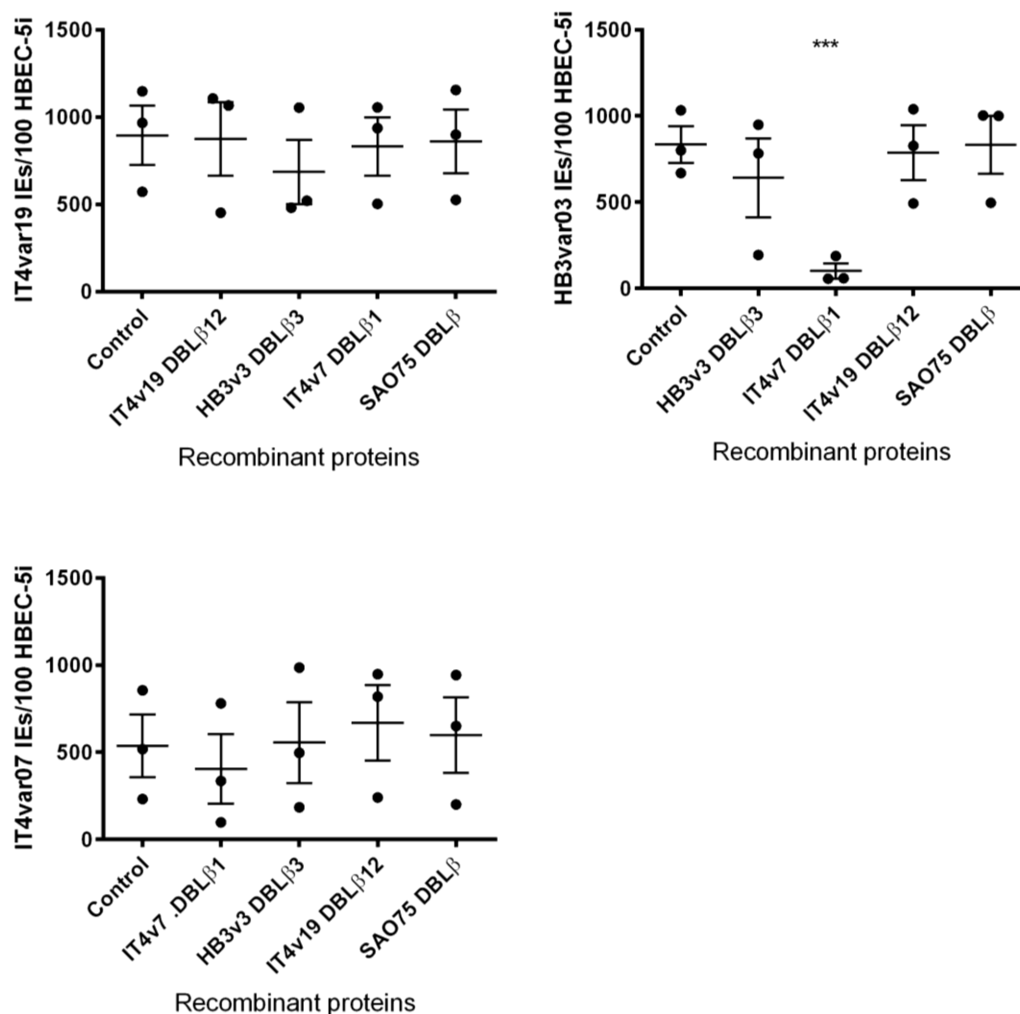


Figure 4.22. Effect of DBLβ recombinant proteins on cytoadhesion of IT4var19, HB3var03 and IT4var07 IEs to HBEC-5i. HBEC-5i were pre-incubated with 50 μg/ml of the DBLβ recombinant proteins in 0.1% BSA/DMEM binding medium before co-incubation with gelatin purified IEs (parasitaemia of at least 30%) for binding. Data shown are the mean and SEM of three experiments, each with duplicate wells. The log transformed values of the number of IEs bound were compared to that of the controls using repeated measures one-way ANOVA with Dunnett's multiple comparisons test (n = 3). No statistically significant reductions in binding were seen.

Recombinant proteins to the didomains, NTS.DBLα1.7-CIDRα1.4 and CIDRα1.4-DBLβ3 of HB3var03 and CIDRα1.1-DBLβ12 of IT4var19, made were of the expected molecular weights. The HB3var03 didomain proteins

(NTS.DBLα1.7- CIDRα1.4 and CIDRα1.4-DBLβ3), however, had a lot of non-specific bands (Figure 4.23), therefore, although they inhibited adhesion in all the parasite lines tested (Figure 4.24), the inhibitory effect cannot be attributed to the didomains as it could be due to impurities.

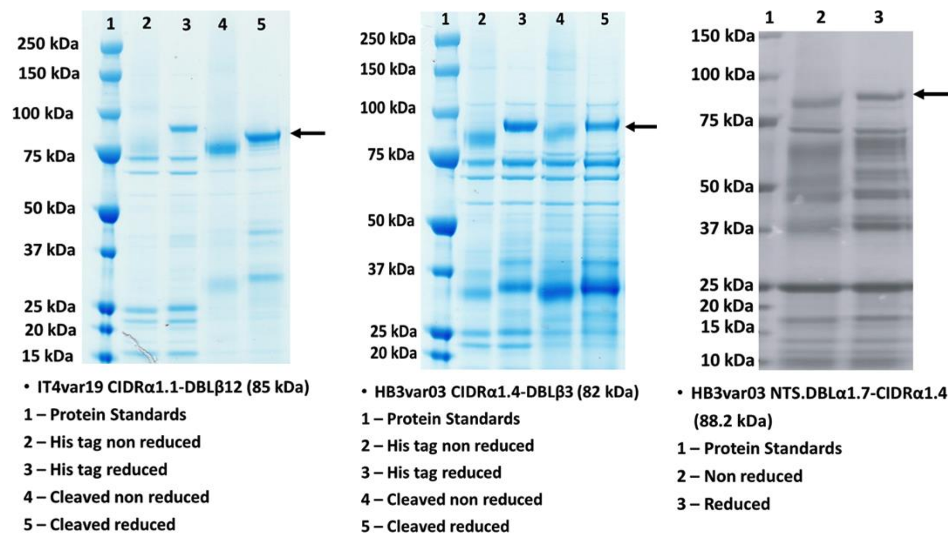


Figure 4.23. SDS-PAGE of recombinant CIDRα1-DBLβ domain proteins of IT4var19 and HB3var03, and NTS.DBLα1-CIDRα1 of HB3var03 expressed in *E. coli*. A gel electrophoresis of 2 µg each of the reduced and non-reduced pairs of the proteins was run on 10% SDS-polyacrylamide proteins. With the exception of the HB3var03 NTS.DBLα1-CIDRα1, proteins before (‘His tag’) and after (‘cleaved’) cleavage of the His tag with TEV protease are also shown.

The ITvar19 CIDRα1.1-DBLβ12 recombinant protein significantly inhibited adhesion of the DC8-expressing variant, IT4var19 IEs, but had no effect on adhesion of the DC13-expressing IEs to HBEC-5i (Figure 4.24). The inhibitory effect of CIDRα1.1-DBLβ12 didomain protein on cytoadhesion of IT4var19 IEs may be due to the CIDRα1 domain in the didomain as the DBLβ12 protein had no effect on adhesion to HBEC-5i (Figure 4.22).

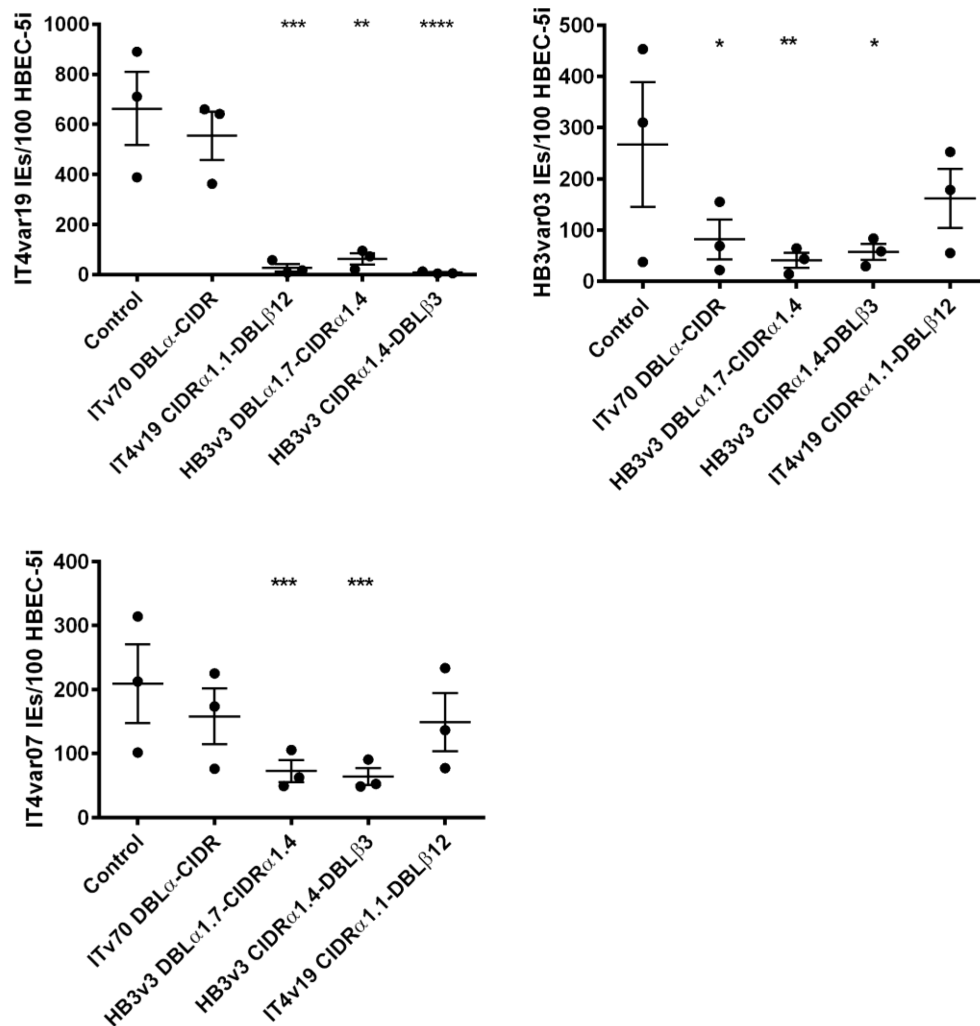


Figure 4.24. Effect of NTS.DBLα-CIDR1α and CIDRα1.1-DBLβ12 recombinant proteins on cytoadhesion of IT4var19, HB3var03 and IT4var07 IEs to HBEC-5i. HBEC-5i were pre-incubated with 50 µg/ml of the recombinant proteins in 0.1% BSA/DMEM binding medium before co-incubation with gelatin purified IEs for binding. Data shown are the mean and SEM of three experiments, each with duplicate wells. The log transformed values of the number of IEs bound were compared to that of the controls using repeated measures one-way ANOVA with Dunnett's multiple comparisons test (n = 3). *P < 0.05. ** P < 0.01. *** P < 0.001. **** P < 0.0001.

4.5.4.2 Effect of PfEMP1 domain antibodies

Antibodies to the NTS.DBLα, CIDRα and DBLβ domains of HB3var03, IT4var07 and IT4var19, the NTS.DBLα-CIDRα (HB3var03 only) and the CIDRα-DBLβ didomain of IT4var19 and HB3var03 (described in section 4.5.4.1) were raised in rabbits (Biogenes Ltd) (Claessens *et al.*, 2012). Although some of these recombinant proteins had non-specific bands on the SDS-PAGE, all the antibodies specifically recognized their homologous IEs and did not recognize heterologous IEs (other parasite lines) when tested in immunofluorescence assays (Rowe *et al.*, unpublished data). Figure 4.25 is an example showing the characteristic punctate staining of the PfEMP1 domain by the antibody while the rabbit IgG (isotype control) did not stain the IEs.

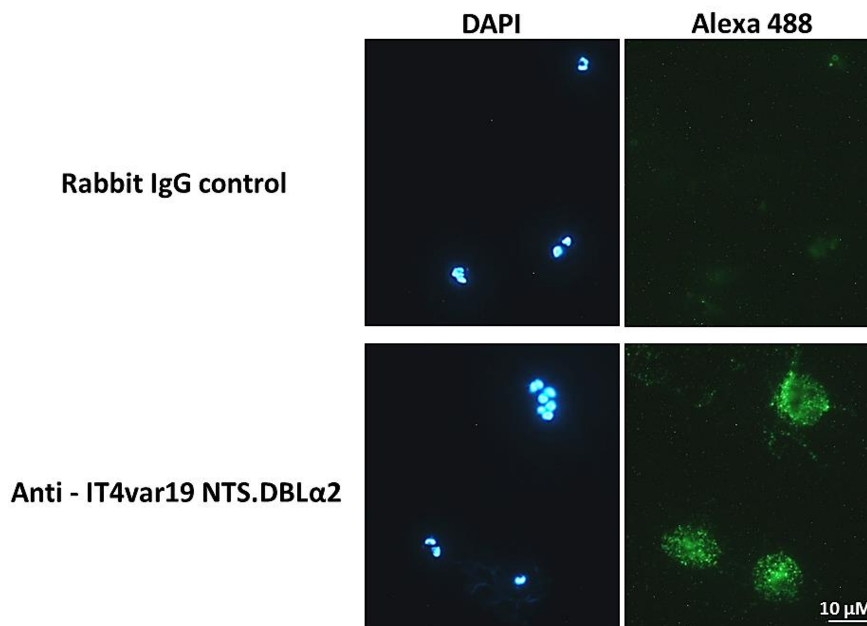


Figure 4.25. Immunofluorescence staining of IT4var19 IEs with antibodies to the NTS.DBLα domain recombinant proteins. Mature pigmented trophozoites were incubated with 20 μg/ml of rabbit antisera to the domain proteins and antibodies bound were detected with 1/1000 Alexa Fluor 488 highly cross-absorbed goat anti-rabbit IgG in DAPI. The green (Alexa 488) fluorescence is positive staining for the PfEMP1 domain on the IEs and the blue fluorescence is DAPI stain of the parasite nuclei.

Only antibodies to the NTS.DBLα domains significantly inhibited adhesion of all the HBEC-binding IEs (Figure 4.26). Antibodies to the CIDRα1 did not affect cytoadhesion of the three parasite lines even though the recombinant protein had been shown to inhibit adhesion of the IT4var19 IEs to HBEC-5i (Figure 4.20). The DBLβ and CIDRα1-DBLβ domain antibodies significantly inhibited the adhesion of IT4var19 and HB3var03 IEs to HBEC-5i. Antibodies to the HB3var03 NTS.DBLα-CIDRα1 also inhibited cytoadhesion of HB3var03 IEs to HBEC-5i. Multiple domains may be involved in the adhesion of the DC8 and DC13 -expressing parasite lines as the DBLβ and CIDRα1.4 of the DC13-expressing HB3var03 and IT4var07 IEs have been reported to bind to ICAM-1 and EPCR respectively (Avril *et al.*, 2016).

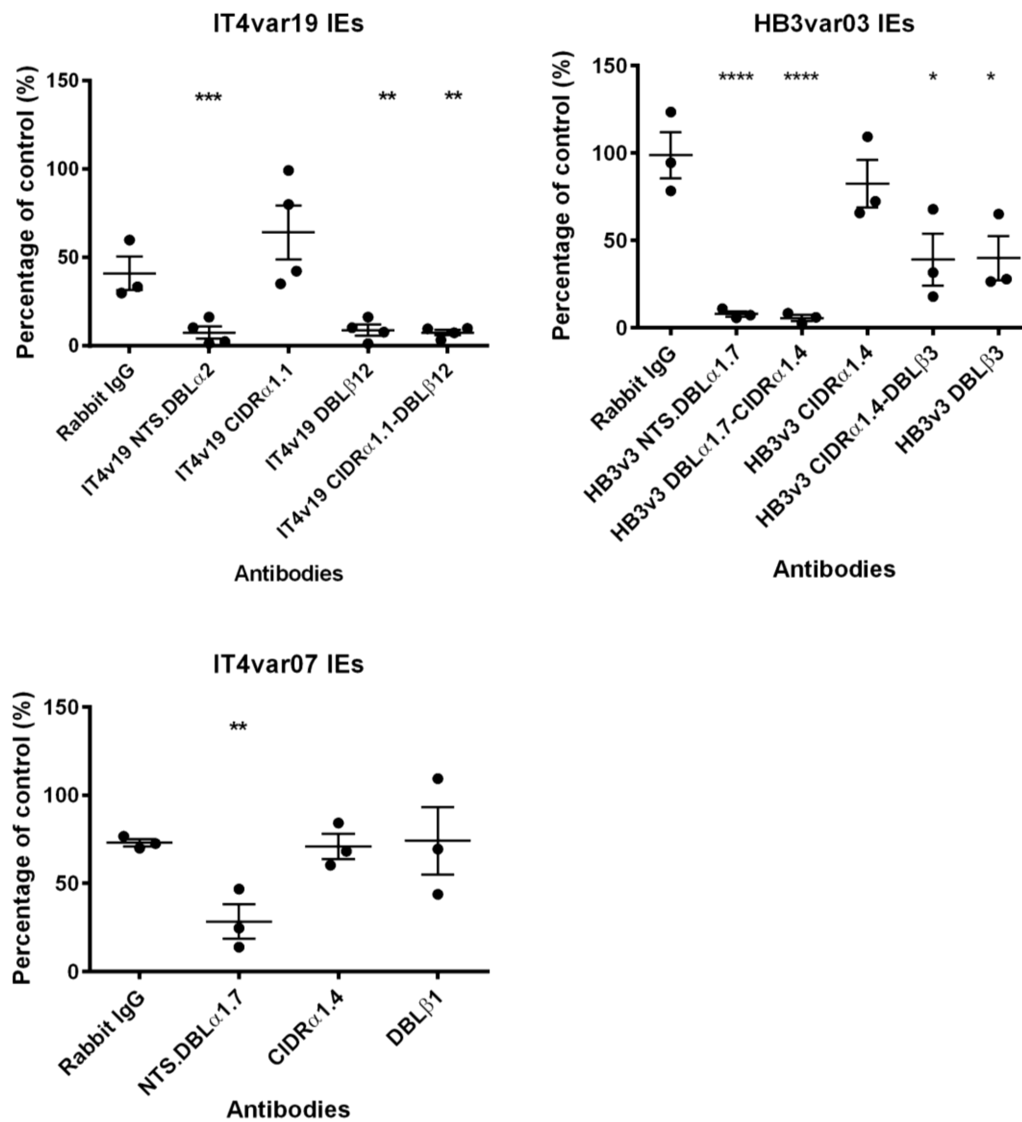


Figure 4.26. Effect of antibodies to PfEMP1 domains of IT4var19, HB3var03 and IT4var07 on cytoadhesion to HBEC-5i. Gelatin purified IEs were pre-incubated with 20 µg/ml of antibodies to the domain proteins before co-incubation with HBEC-5i for binding. Data shown are the mean and SEM of the percentage of IEs bound relative to the control for at least three experiments, each with duplicate wells. The log-transformed values of the number of IEs bound were compared to that of the controls using repeated measures one-way ANOVA with Dunnett's multiple comparisons test ($n \geq 3$). * $P < 0.05$. ** $P < 0.01$. *** $P < 0.001$. **** $P < 0.0001$.

4.6 Discussion

Cerebral malaria is thought to be an organ-specific disease due to the marked sequestration in the brain. Parasite expression of the DC8 and DC13 PfEMP1 encoded by group A-like (B/A and A) *var* genes in IEs that bind to brain endothelial cells may be a major contributing factor to the organ specificity of the disease. In this study the group A-like *var* genes which have been associated with cerebral malaria in children were predominantly transcribed by the two Kenyan parasite lines after selection for binding to HBEC as previously reported using other parasite lines (Avril *et al.*, 2012; Claessens *et al.*, 2012).

The *9197var27* gene predominantly transcribed by the 9197.HBEC encodes a DC8 PfEMP1 and the 8211 DBLα_tag1 also highly transcribed after HBEC-selection, grouped with DC8 PfEMP1, however, the domain cassettes of 8211 PfEMP1 could not be confirmed because the full genome sequence of the parasite line is not yet available. *9197var15* on the other hand, which is a group A variant that encodes a PfEMP1 with a DBLα1.2 domain, could not be classified into any of the domain cassettes of PfEMP1 described so far (Rask *et al.*, 2010). The other two predominantly transcribed tags in the HBEC-selected 8211, DBLα_tag 18 and 19 both expressed DBLα1.2 domains similar to that identified in 9197var15. These data, therefore, show that other non- DC8 and DC13 genes that express DBLα1.2-CIDRα1 encoding *var* genes also mediate adhesion to HBEC.

The association of *var* genes that encode DC8 and DC13 PfEMP1 with cerebral malaria, although not yet well established, may fit with a *var*-specific disease as it has been established for *var2csa* in pregnancy associated malaria (PAM) (Salanti *et al.*, no date; Ndam *et al.*, 2005; Fried and Duffy, 2015; Travassos *et al.*, 2015), however, these group A-like DC8 and DC13 *var* genes were observed to be highly transcribed by parasites in peripheral blood from children with cerebral malaria and other types of severe malaria including respiratory distress (Lavstsen *et al.*, 2012). Other studies that looked at cerebral malaria, in comparison to uncomplicated malaria, had no other severe malaria controls (Bertin *et al.*, 2013; Almelli *et al.*, 2014; Tembo *et al.*, 2014). *In vitro*

studies also show that the DC8 and DC13 -expressing IEs can bind to diverse endothelial cells including the brain endothelial cells (Avril *et al.*, 2012, 2013; Claessens *et al.*, 2012; Gillrie *et al.*, 2015). These data do not seem to support the role of the DC8 and DC13 *var* types in the brain-specific sequestration found in cerebral malaria. It would be interesting to investigate whether parasite expression of DBLα1.2-CIDRα1 domain cassette may be associated with cerebral malaria. Other mechanism (s) in the brain could also promote increased binding in brain that is absent in other organs. Cytokines are implicated in the pathology of cerebral malaria (reviewed in (reviewed in Crompton *et al.*, 2014) and may play such a role. The proinflammatory cytokine, Interleukin (IL)-1β, was found to be expressed in the brain of children who died of cerebral malaria but not in normal brain controls (Brown *et al.*, 1999; Hunt and Grau, 2003; Armah *et al.*, 2005), and this cytokine may increase expression of the receptor (s) in the brain as have been documented for TNFα induced ICAM-1 upregulation on endothelial cells (O'Carroll *et al.*, 2015).

The Group A-like *var* genes appear to have diverged from groups B and C, in sequence and adhesion properties (Robinson, Welch and Smith, 2003), hence, though the CIDRα (2-6) of groups B and C bind to CD36, the CIDRα1/β/δ/γ of group A do not. The CIDRα1 domain of the Group A-like DC8 and DC13 PfEMP1 has been identified as the domain binding to EPCR on human brain endothelial cells (Turner *et al.*, 2013; Lau *et al.*, 2015). However, using the DC8 and DC13 -expressing IEs and HBEC-5i, the CIDRα1 recombinant proteins blocked binding of only IT4var19 IEs to HBEC-5i and had no effect on cytoadhesion of the two DC13-expressing IEs suggesting that other domain(s) may mediate adhesion of the DC13-expressing IEs to HBEC and that binding to EPCR may not be required for all adhesion of all the HBEC-binding IEs.

The DBLβ domains of DC8 and DC13 PfEMP1 may also provide evidence to support the divergence of the group A-like *var* genes from other group A (DC4), B and C which express DBLβ that bind to ICAM-1 (Janes *et al.*, 2011; Bengtsson *et al.*, 2013). However, HBEC-binding of HB3var03 and IT4var07 IEs have been suggested to be also mediated by the first DBLβ domain via binding to ICAM-1 (Avril *et al.*, 2016). With the exception of inhibition of cytoadhesion

of HB3var03 IEs by IT4var07 DBLβ recombinant protein, binding of the DBLβ recombinant proteins to HBEC had no effect on cytoadhesion of the DC8 and DC13 -expressing parasite lines tested. Avril *et al.* (Avril *et al.*, 2013), tested the effect of the CIDRα1 and DBLβ12 recombinant proteins on cytoadhesion of IT4var19 to THBMEC and had similar results showing inhibition of adhesion with the CIDRα1 and no effect with the DBLβ recombinant proteins or the CIDRβ and DBLγ6/9 domains towards the C-terminal. Antibodies to the recombinant domains proteins that contained the DBLβ domain, however, significantly inhibited cytoadhesion of IT4var19 and HB3var03 IEs to HBEC-5i. This suggests that although the DBLβ domain may not be directly involved in the binding of the IEs to HBEC, binding of domain antibodies to the PfEMP1 on the IEs could interfere with the adhesion of the IEs to HBEC-5i.

Antibodies to the NTS.DBLα domains also inhibited adhesion of all the DC8 and DC13 -expressing parasite lines, although the recombinant proteins had no effect on the cytoadhesion. The DBLα2 recombinant protein of the DC8 PfEMP1 expressing parasite line IT4var19 have been reported not to affect the cytoadhesion of the IT4var19 IEs to THBMEC (Avril *et al.*, 2012) while data shown here and by others (Avril *et al.*, 2013; Turner *et al.*, 2013) show that the CIDRα1 domain mediates adhesion of the DC8-expressing parasites. The differences between the effect of the recombinant proteins and their respective antibodies may be due to interference of binding of the recombinant proteins by the aggregates and fragments in the proteins produced. It is also possible that, as suggested for DBLβ domains, the binding of antibodies to the NTS.DBLα of the DC8 and DC13 PfEMP1 on the IEs may be sufficient to obstruct adhesion of the IEs to HBEC irrespective of the binding domain. While the data does not show that the NTS.DBLα mediates adhesion of the IEs to HBEC, they show that the inhibitory effect of the NTS.DBLα antibodies could be explored further for potential cytoadhesion blocking therapies.

The possibly conserved DBLα1.2/1.7/2-CIDRα1 domains of the IEs that bind to HBEC, may be responsible for binding to HBEC. The role of other domains within the DC8 and DC13 PfEMP1 towards the C-terminal, need to be investigated in detail with the IEs and HBEC, in order to test the hypothesis that

the NTS.DBLα domain may mediate adhesion of the DC13-expressing parasite lines while the CIDRα1 mediates that of the DC8-expressing parasites. This needs to be explored further by optimizing the production of DC8 and DC13 DBLα and other domain recombinant proteins to generate good quality proteins and or by genetic manipulation of the expression of the domains for HBEC-binding assays.

5 Chapter V: The role of EPCR and other host receptors in mediating adhesion to HBEC

5.1 Abstract

Endothelial Protein C Receptor (EPCR), a transmembrane glycoprotein also found in plasma has been implicated in the pathology of severe malaria. EPCR binds protein C to give cytoprotective effects and it is hypothesized that the binding of IEs to EPCR may interfere with protein C binding and hence inhibit its protective functions, contributing to pathology of severe malaria. IEs that express PfEMP1 of Domain Cassettes (DC) 8 and 13 bind to Human Brain Endothelial Cells (HBEC) and it has been suggested that these DC8 and DC13 -expressing parasites bind to EPCR on HBEC to contribute to sequestration in the microvasculature of brain and the resulting pathology of cerebral malaria.

P. falciparum strains, HB3 and IT4 expressing HB3var03 (DC13), IT4var07 (DC13) or IT4var19 (DC8) PfEMP1 were used to investigate the role of EPCR in HBEC-binding. Static and flow binding assays, and live immunofluorescence assays showed that parasites expressing IT4var19 (DC8) bind to EPCR. However, parasites expressing HB3var03 (DC13) or IT4var07 (DC13), did not bind EPCR. Furthermore, EPCR antibodies, soluble protein and EPCR siRNA knockdown inhibited adhesion of IT4var19 IEs to HBEC by >80%, but had no significant effect on binding of the other two parasite lines. These data show that not all DC8 and DC13 -expressing IEs bind to EPCR, even though recombinant HB3var03 and IT4var07 proteins had previously shown high affinity EPCR-binding. This suggests that additional receptors, other than EPCR, are required for HBEC-binding.

5.2 Introduction

Severe forms of malaria include severe anaemia, respiratory distress, PAM and cerebral malaria. Differences in the pathology of severe malaria may be due to specific host factors such as immune response, polymorphisms or expression of certain receptors in particular organs, or parasite factors, which may include growth rates and the expression of specific antigens.

P. falciparum expression of variable surface antigen, PfEMP1, on the surface of IEs is thought to enable binding host cells to cause the marked sequestration seen in the brain and placenta of cerebral malaria and PAM respectively. For cerebral malaria, parasite that express group B/A or A PfEMP1 containing DC8 and DC13 bind to HBEC and these groups of PfEMP1 are predominantly transcribed in children with severe malaria (Claessens *et al.*, 2012; Lavstsen *et al.*, 2012; Avril *et al.*, 2013; Bertin *et al.*, 2013; Almelli *et al.*, 2014b).

IEs have been shown to bind to a variety of host receptors including ICAM-1 (Berendt *et al.*, 1989; Ochola *et al.*, 2011), CD36 (Barnwell *et al.*, 1989; Ockenhouse *et al.*, 1991), PECAM-1, (Heddini et al. 2001), HABP-1 (Biswas *et al.*, 2007), CSA (Rogerson *et al.*, 1995) and complement receptor 1 , CR1 (Rowe *et al.*, 1997; Krych-Goldberg, Moulds and Atkinson, 2002). Binding to these receptors do not always lead to severe disease (Snow and Marsh, 1998), however, as it has been described for sequestration of IEs in the placenta via CSA, cytoadherence in certain tissues or organs such as the brain may lead to severe pathology of the disease.

Adhesion to ICAM-1 has been implicated in cerebral malaria (Turner *et al.*, 1994; Newbold *et al.*, 1997; Ochola *et al.*, 2011) however, there is conflicting data about its role as a receptor for IE binding in the brain in cerebral malaria (Ockenhouse *et al.*, 1991; Rogerson *et al.*, 1999; Andreas Heddini *et al.*, 2001) or its role in HBEC-binding by the DC8 and DC13 -expressing parasites (Avril *et al.*, 2012, 2016; Claessens *et al.*, 2012; Turner *et al.*, 2013). Instead, recent work suggests that EPCR may be the most important receptor in cerebral malaria by

mediating adhesion of the DC8 and DC13 -expressing IEs to HBEC (Turner *et al.*, 2013).

EPCR is a transmembrane glycoprotein that is also found in plasma in its soluble form, sEPCR (Rao, Esmon and Pendurthi, 2014). EPCR on interaction with the thrombin-thrombomodulin complex binds to circulating protein C to give its activated form. Activated protein C (APC) via the cleavage of protease activated receptor 1 (PAR1), elicits anti-coagulative, anti-inflammatory and cytoprotective effects, including membrane barrier protection, on endothelial cells (Riewald *et al.*, 2002; Bouwens, Stavenuiter and Mosnier, 2013). In the brain, APC has been associated with neuroprotective effects (Deane *et al.*, 2009) and implicated in the prevention of apoptosis in the endothelium (Cheng *et al.*, 2003). It is therefore hypothesised that the binding of IEs to EPCR may interfere with binding to protein C and hence abrogate the protective effects of APC, resulting in the excessive release of proinflammatory cytokines, increased permeability or damage to the blood brain barrier among other pathologies associated with cerebral malaria.

The IEs are suggested to bind to EPCR via the CIDRα 1.1 and 1.4 of the DC8 and DC13 PfEMP1 variants although CIDRα1.5-1.8 of others types of PfEMP1 also bind to EPCR (Turner *et al.*, 2013; Lau *et al.*, 2015). Individuals in malaria endemic areas have also been reported to acquire antibodies to these CIDRα1 types early in life (Turner *et al.*, 2015). Most of these studies have, however, been done with recombinant proteins (i.e. a full-length PfEMP1 or CIDRα1 recombinant proteins and EPCR) and detailed studies of interaction of EPCR with IEs and HBEC are currently lacking. There is also limited data on the role of the other host receptors in the adhesion of the DC8 and 13 -expressing parasites to HBEC. Therefore, EPCR and other host receptors were investigated using recombinant proteins and antibodies together with the DC8 and 13 -expressing parasites to elucidate their involvement in adhesion to HBEC.

5.3 Hypothesis and specific aims

The hypothesis that EPCR is the receptor mediating binding of DC8 and DC13 -expressing IEs to HBEC-5i, was investigated in this chapter.

Specific aims

1. To test HBEC-binding IEs for binding to recombinant EPCR protein and other receptor molecules
2. To determine the effect of EPCR recombinant protein and antibodies, and other receptor recombinant proteins on cytoadhesion
3. To determine the effect of EPCR knock down on adhesion to HBEC-5i
4. To confirm that binding of IEs to EPCR can be inhibited by DC8 and DC13 CIDR1α domains
5. To investigate whether soluble EPCR in serum can inhibit IE-binding to HBEC-5i

5.4 Methods

5.4.1 Binding of IEs to receptor molecules

Recombinant human proteins, EPCR (Sino Biologicals, 13320-H02H), ICAM-1 (R&D 720-IC), CD36 (R&D systems, 1955-CD), PECAM-1, (Sino Biologicals, 10148-H02H), NCAM-1, (R&D systems, 2408-NC), HABP-1, (R&D systems, 5155-HB), VCAM-1, (R&D systems, 862-VC), and purified glycosaminoglycans; heparin sodium salt from porcine intestinal mucosa (Sigma-Aldrich, H4784), hyaluronic acid sodium salt from bovine vitreous humor (Sigma-Aldrich, H7630) or CSA from bovine trachea (Sigma-Aldrich, 27042) were diluted to 50 µg/ml in PBS. Three µl of the diluted recombinant protein was spotted on two untreated Petri dish (BD Falcon, 351007), two spots per dish in a radial fashion at an equal distance from the center and incubated overnight at 4°C. The

proteins were aspirated and the plate was blocked with 2 ml 3% BSA/PBS for 2 hours at 37°C.

The 3% BSA/PBS was taken off and the dish was washed with 2 ml of DMEM binding medium by swirling the medium in the plate and taking the medium off to be replaced with 2 ml of fresh DMEM binding medium for a second wash. Gelatin purified pigmented trophozoites, at not less than 30% parasitaemia, were washed twice with DMEM binding medium and made up to 2% haematocrit in 0.1% BSA/DMEM binding medium. Two ml of the IEs suspension was added to each dish and the dishes were incubated at 37°C in air for 1 hour, with resuspension every 12 minutes. The dish was washed gently by taking off the IE suspension and adding DMEM binding medium. The dish was gently swirled four directions, clockwise and anticlockwise and the medium was taken off and the wash repeated with regular checks under an inverted microscope till there were no more unbound cells. The adherent IEs were fixed with 2% glutaraldehyde in PBS for 1 hour, and stained with 5% Giemsa in Giemsa buffer (PBS pH 7.2) for 10 minutes. Bound IEs on 3 fields were counted for each spot with a light microscope using the 40X objective on the Olympus CK2 inverted microscope.

5.4.2 Immunofluorescence staining of protein bound IEs

For the IFA, the assay was done as above (section 5.4.1) but three or four spots of 5 µl of the diluted protein were spotted onto one untreated Petri dish and 5 ml of IEs at 1% haematocrit was added to each dish. After incubation, the dish was washed but the cells were not fixed but washed twice with PBS. Spots were drawn around the bound IEs on the dish with a Super Mini Pap Pen (Thermo Fisher Scientific, 00-8877), a water repellent pen that creates a barrier to retain the antibodies within the spots for staining the parasites.

Fifty µl of 20 µg/ml (diluted in 1% BSA/PBS) of rabbit antisera to the NTS-DBLα1 of the PfEMP1 of interest (Claessens *et al.*, 2012) or rabbit IgG (isotype control), and 1% BSA/PBS (“no antibody” control) was added to a spot. The dish was incubated for 30 minutes in a humid box at room temperature.

The dish was first washed with 5 ml PBS, which was taken off after swirling the dish. The wash was repeated twice by adding 5 ml of PBS and taking the PBS off after 5 minutes for each wash. Then 50 µl of 1: 500 Alexa Fluor 488 goat anti-rabbit IgG in 1 µg/ml DAPI was added to each spot and incubated for 30 minutes in a dark humid box.

The dish was washed as described above and air-dried. A drop of fluoromount (Sigma-Aldrich, F4680) was added to each spot and covered with a coverslip. The dish was stored at 4°C till ready for viewing at 1000X under a fluorescence microscope and pictures were taken with the YenCam camera.

5.4.3 Flow binding assay with recombinant EPCR protein

A µ-Slide I 0.8 Luer (ibidi, 80191) was coated with 50 µg/ml recombinant EPCR (a gift from Prof. Matt K. Higgins) in PBS and incubated in a humid box for 2 hours at 4°C. The EPCR was carefully removed, replaced with 1%BSA/PBS (blocking buffer) and incubated overnight in a humid box at 4°C for use the next day. On the day of the assay, the blocking buffer was gently taken off and the coated µ-Slide was washed twice with DMEM binding medium. IEs at pigmented trophozoite stage at not less than 30% parasitaemia were also washed twice in DMEM binding medium and made up to 1% haematocrit in 0.1%BSA/DMEM binding medium. The ibidi pump system was set up and the assay continued with the IEs and the EPCR-coated µ-Slide as described in section 3.48, with the same flow settings of a unidirectional flow with a switching rate of 5 seconds at a sheer stress of 1 dyne/cm^2.

5.4.4 Static HBEC-5i or HBMEC binding inhibition assays with host receptor molecules

Recombinant protein inhibition assay

Binding assays were carried out as described in sections 2.4 and 2.5, with a 20-minute pre-incubation step. Gelatin purified IEs at 30-80% parasitaemia were

washed and suspended at 2% hematocrit in 190 µl of 20 µg/ml of EPCR (a gift from Prof. Matt K. Higgins), CD36, PECAM-1 or HABP-1 in 0.1% BSA/DMEM binding medium. The IEs with recombinant proteins were incubated for 20 minutes with a resuspension by flicking the tubes after 10 minutes. After the incubation, 190 µl of the suspension was added to the HBEC-5i or HBMEC in each well and the cells were co-incubated for one hour. Binding was compared with control that contained IEs and HBEC-5i in 0.1% BSA/DMEM binding medium only.

Antibody inhibition assay

HBEC-5i in the wells were washed twice and 190 µl of rat monoclonal antibody to human EPCR, RCR-252 (HM2145, Hycult), goat polyclonal antibody to human EPCR (R&D systems, AF2245), rat IgG_1 (Hycult, HI3001) or goat IgG (R&D systems, AB-108-C) at 20 µg/ml in 0.1% BSA/DMEM binding medium was added to each well. The HBEC-5i with the antibodies were incubated for 20 minutes with a resuspension by swirling after 10 minutes of incubation. IEs were added to the HBEC-5i to make up 2% haematocrit in each well and the cells were co-incubated for one hour. Binding was compared with control wells that contained IEs and HBEC-5i in 0.1% BSA/DMEM binding medium only.

5.4.5 Transfection of HBEC-5i with EPCR siRNA

HBEC-5i were seeded onto 0.1% gelatin coated wells in a 48-well plate (for binding assay) or 75 cm^2 flasks (to determine the success of the EPCR knockdown on the surface of the HBEC-5i), at least, one day before the assay, to reach 60 to 80% confluency on the day of transfection. Lipofectamine RNAiMAX transfection reagent (Thermo Fisher Scientific, 13778030) and 30 nmol of EPCR siRNA (Santa Cruz, sc-39932), control A siRNA (Santa Cruz, sc-37007), a scrambled sequence that does not cause a specific effect in the cell or has no known target in the cell or Control A siRNA- FITC (Santa Cruz, sc-36869) (used to check transfection efficiency) were each diluted with Opti-MEM reduced serum medium (Thermo Fisher Scientific, 31985062) in a ratio 3:50.

An equal volume of the diluted EPCR siRNA, control A siRNA or Control A siRNA-FITC was added to the diluted Lipofectamine RNAiMAX transfection reagent and incubated at room temperature for 5 minutes to form the siRNA-lipid complex. The medium on the HBEC-5i was aspirated and 50 µl of the siRNA-lipid complex was added to 300 µl of complete DMEM per well on the 48-well plate, and for the 75 cm^2 flask, 1.5 ml of the siRNA-lipid complex was added to 8.5 ml of complete DMEM medium on the cells. The cells were incubated in 5% CO_2 at 37°C for 48 hours. The transfected cells in the 48-well plate were then used for binding assays and those in the flask used for flow cytometry to determine the effect of the transfection on EPCR expression.

5.4.6 Flow cytometry to determine EPCR expressed on surface of HBEC-5i

The media on HBEC-5i (not transfected) and EPCR-siRNA, control A siRNA or Control A siRNA-FITC -transfected HBEC-5i was aspirated 48 hours after transfection and the cells were washed twice with 10 ml PBS. The cells were lifted by adding 5 ml of 4 mg/ml lidocaine hydrochloride (AppliChem, A2870) in PBS after which they were incubated for 5 to 10 minutes at 37°C. Ten ml of PBS was added to recover the cells and the suspension was centrifuged at 800g for 4 minutes. The supernatant was aspirated and the cells were fixed with 4 ml of 2 % (v/v) formaldehyde (Thermo Fisher Scientific, 28906) in PBS for 10 minutes at room temperature. The fixed cells were transferred to 4 FACS tubes, 1 ml each, and centrifuged at 3500g for 4 minutes. The supernatant was decanted, and the cells were washed with 2 ml cold FACS buffer.

One hundred and twenty µl of primary antibody, 1 µg/ml of goat polyclonal antibody to human EPCR or goat IgG (isotype control) and 0.5 µg/ml of mouse monoclonal antibody to human ICAM-1 (AbD Serotec, MCA1615XZ) or mouse IgG, isotype control (Sigma-Aldrich, MOPC-31c), in 1.25 µg/mL Hoechst/ PBS was each added to the cells and incubated at 4°C for an hour with the tubes flicked every 10 minutes. The cells were washed with 3 ml of cold FACS buffer (0.1% BSA/0.1% azide in PBS) and the supernatant aspirated.

One hundred and fifty µl of Alexa Fluor 488 donkey anti-goat (Thermo Fisher Scientific, A-11055) or Alexa Fluor 488 goat anti-mouse (Thermo Fisher Scientific, A-11029) at 1:1500 in 1.25 µg/mL Hoechst/ PBS was added to the cells in each tube and incubated at 4°C in the dark for an hour with the samples resuspended every 10 minutes. The cells were washed with cold FACS buffer, the supernatant was aspirated and the cells resuspended in 250 µl of cold FACS buffer. The samples were analyzed immediately on a Becton-Dickinson LSRII flow cytometer or stored at 4°C and analyzed within 24 hours as described in section 2.8.

5.4.7 Depletion of EPCR in human serum

Pooled human serum (from donors at Scottish National Blood Transfusion Service) was depleted of EPCR to determine if soluble EPCR in serum was inhibiting adhesion of IEs to HBEC-5i. The EPCR depletion was done using EPCR-antibody coated Dynabeads M-450 Epoxy (Thermo Fisher Scientific, 14011). Seventy-five µl of Dynabeads (hereafter referred to as 'beads') was washed with 150 µl of 0.1% sodium phosphate buffer pH 7.6 (0.845 M Na_2HPO_4 and 0.155 M NaH_2PO_4 in dH_2O) and mixed by inversion.

The tube was placed in a magnet, Dynal MPC-50 Magnetic Stand (Ambion, 10025), for 1 minute and the supernatant was discarded. The wash was repeated and the beads were resuspended in 50 µl of the 0.1% sodium phosphate buffer. Goat polyclonal antibody to human EPCR was added to the beads to a final antibody concentration of 133 µg/ml. The suspension was incubated with gentle rotation for 15 minutes at room temperature on a vertical rotator (Glas-Col LLC, friction-drive test tube head) at 25rpm after which 0.1% BSA was added. The suspension was put back on the vertical rotator for 16 to 24 hours.

The next day, the tube was placed in the magnet for 1 minute and the supernatant discarded. The coated beads were washed with 300 µl of 0.1% BSA/PBS pH7.4 and put back on the magnet. The supernatant was aspirated and the wash was repeated, followed by resuspension of the beads in 150 µl of 0.1%

BSA/PBS. The suspension was divided into two tubes, 75 µl each. One tube was placed on the magnet, the supernatant taken off and the beads were suspended in 200 µl of pooled human serum. The suspension was incubated on the vertical rotator at room temperature for 45 minutes and placed on the magnet. The other tube with the coated beads in 0.1% BSA/PBS only was also placed on the magnet and the supernatant discarded.

The supernatant from the tube containing serum was gently pipetted into the tube with EPCR antibody coated beads only, to ensure that any EPCR remaining in the serum was removed by binding to the EPCR antibody coated beads. The suspension was incubated on the vertical rotator at room temperature for 45 minutes and placed on the magnet. The supernatant was gently pipetted in to fresh tubes. Aliquots of the EPCR-depleted human serum were stored at -20°C along with control aliquots of normal (non-depleted) human serum.

5.4.8 ELISA to measure EPCR in human serum

A sandwich ELISA was done to determine the success of the depletion of EPCR in human serum and also to determine the amount of EPCR in the pooled human serum. The wells of a 96-well microtiter plate were each coated with a capture antibody, 100 µl of 1.0 µg/ml rat monoclonal antibody to human EPCR in PBS and incubated overnight at 4°C on two wet tissues and wrapped in cling film. The unbound antibody was removed by washing 4 times with 0.05% Tween-20 (Sigma-aldrich, P9416)/PBS (PBST) by the Skatron Skanwasher 300. The plate was blotted dry on 3 ply hand towels (Kimberley Clark, 6771). The wells were blocked with 200 µl of 1% (w/v) skimmed milk powder in PBST (blocking buffer) and incubated at room temperature for 3 hours. The plate was washed 4 times with PBST and blotted dry on 3 ply hand towels.

Recombinant human EPCR (a gift from Prof. Matt K. Higgins) was serially diluted in blocking buffer to give the following concentrations in ng/ml; 0.31, 0.63, 1.25, 2.50, 5.00. Pooled human serum and EPCR-depleted human serum were also diluted 1 in 10 with blocking buffer. One hundred µl each of the

diluted serum, EPCR recombinant protein and 5 ng/ml of CD36 in blocking buffer, or blocking buffer only was added to duplicate wells and incubated at room temperature for 2 hours on two wet tissues and wrapped in cling film. The plate was washed 4 times with PBST and blotted dry on 3 ply hand towels.

The detection antibody, 100 µl of 0.5 µg/ml EPCR polyclonal antibody in blocking buffer was added to each well and the plate was incubated at room temperature for 2 hours in humid conditions described above. The plate was washed 4 times with PBST, blotted dry on 3 ply hand towels and 100 µl of 1/3000 horseradish peroxidase conjugated anti-goat IgG in PBST added to each well. The plate was incubated in humid conditions for 1 hour at room temperature. The unbound secondary antibody was removed by washing of the plate 4 times with PBST and blotting of the plate dry. The reaction was developed with 100 µl of substrate buffer (0.04 mg/ml o-Phenylenediamine dihydrochloride, OPD (Sigma- Aldrich, P8787) and 0.012% v/v H_2O_2 in 24.5 mM citric acid monohydrate and 52 mM Na_2HPO_4, pH 5.0). The OPD tablet and H_2O_2 were added immediately before use, as OPD is light sensitive. The plate was incubated at room temperature for 15 minutes and the reaction stopped by the addition of 25 µl of 2.0 M H_2SO_4 to each well. The optical density per well was read at 492 nm using the Labsystems Multiskan Ascent microtiter plate reader.

5.4.9 HBEC-5i binding assay with EPCR-depleted human serum

The assay was done as described in section 3.4.6. Briefly, gelatin pigmented trophozoites of at least 30% parasitaemia were suspended at 2% haematocrit in 10% EPCR-depleted human serum in DMEM binding medium, normal (non-depleted) human serum in DMEM binding medium or 0.1% BSA /DMEM binding medium, and 200 µl of the parasite suspension added per well to the HBEC-5i. The cells were incubated at 37°C for 75 minutes with gentle rocking of the plate every 30 minutes. The unbound IEs were gently washed off, the cells were fixed and the number of IEs bound to at least 100 HBEC-5i was counted.

5.5 Results

5.5.1 Binding to EPCR and other receptor molecules

From both static and flow protein binding assays, only IT4var19 IEs bound to EPCR recombinant protein (Figure 5.1 and Figure 5.2). The two DC13-expressing HB3var03 and IT4var07 IEs did not bind to EPCR and neither did 9197.HBEC under static conditions.

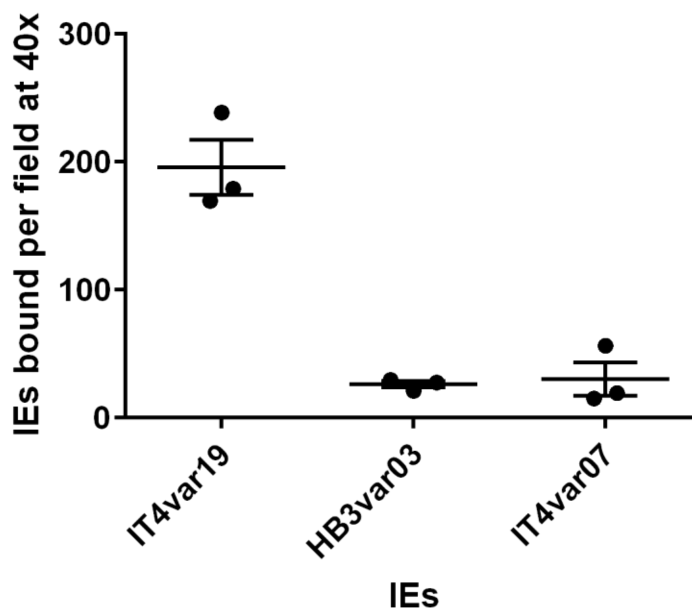


Figure 5.1. Binding of IT4var07, IT4var19 and HB3var03 IEs to recombinant EPCR under flow conditions. Gelatin purified pigmented trophozoites, of at least 30% parasitaemia, were flowed over a µ-slide coated with 50 µg/ml of EPCR under parallel flow conditions at shear stress of 1 dyne/cm^2. After 5 minutes, IEs bound per 10 fields were counted at 400x, under continuous flow conditions. Data shown are the mean and SEM of three experiments (n = 3).

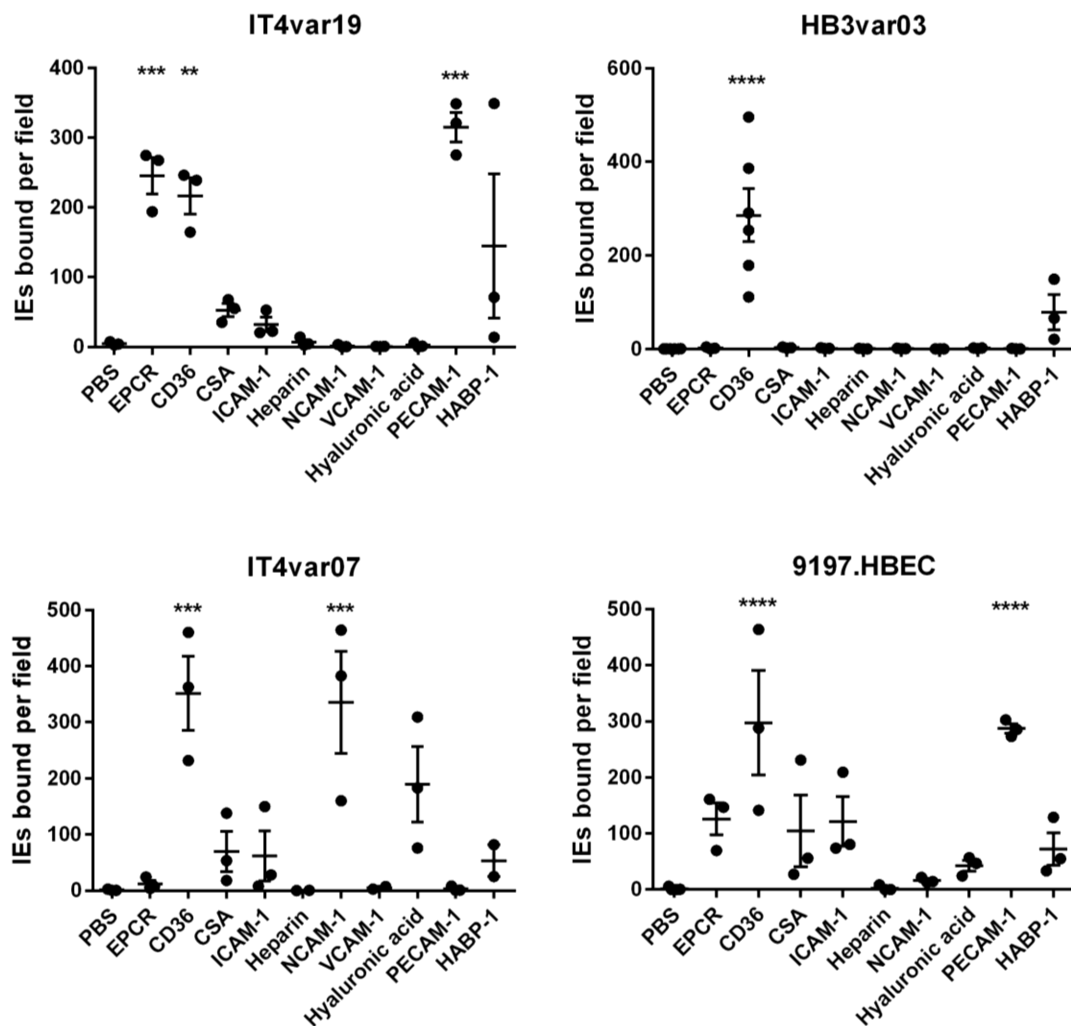


Figure 5.2. Binding of IT4var07, IT4var19 and HB3var03 IEs bind to recombinant receptor proteins and glycosaminoglycans under static conditions. Gelatin purified IEs, of at least 30% parasitaemia, in 0.1% BSA/DMEM binding medium were incubated with 50 µg/ml of protein absorbed on a dish for an hour at 37°C. Each experiment included duplicate dishes, with two spots per receptor on a dish, and IEs were counted for three fields per spot at 400X. The data shown are mean and SEM of at least three independent experiments. The means were compared to the mean of the PBS controls using repeated measures one-way ANOVA with Dunnett's multiple comparisons test, $n \geq 3$; ** $P < 0.01$, *** $P < 0.001$ and **** $P < 0.0001$.

Although the IEs used for the experiments were regularly enriched by FACS or panning on HBEC-5i for the expression of PfEMP1 of interest, the PfEMP1 expression of the parasites in the cultures used is not mono-variant due

to spontaneous *var* gene switching. Hence there was always a sub-population of parasites in the culture expressing other PfEMP1 variants. Therefore, the IEs bound to the proteins were stained with antibodies to the NTS.DBLα to determine if they expressed the PfEMP1 of interest. IEs bound to EPCR from the IT4var19 culture were confirmed to be expressing IT4var19 (Figure 5.3).

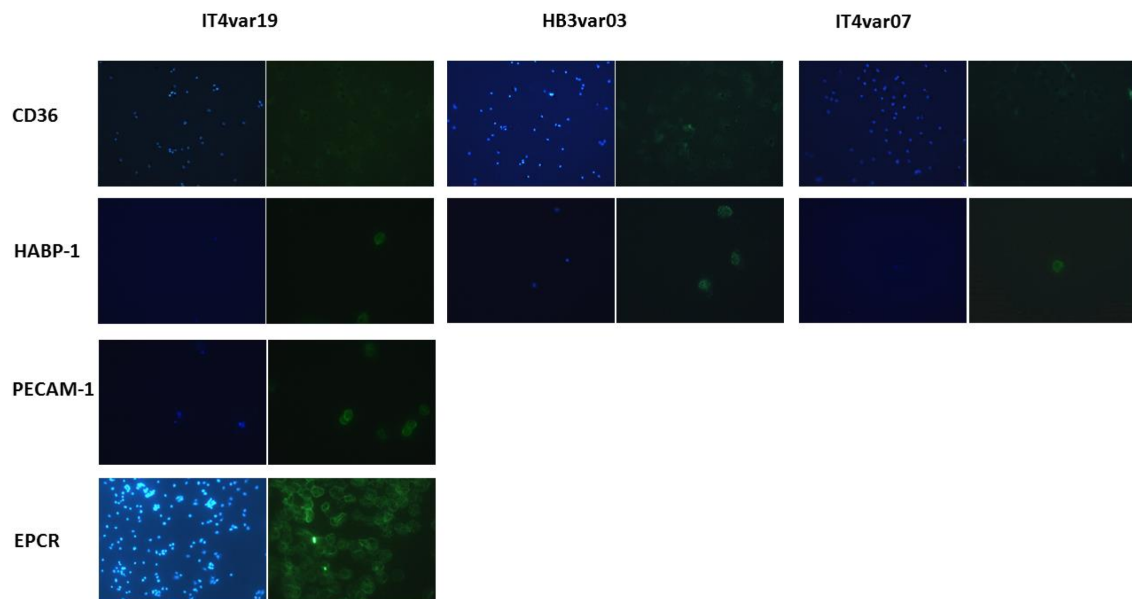


Figure 5.3. PfEMP1 antibody staining of IT4var19, HB3var03 and IT4var07 IEs bound to receptor recombinant proteins. IEs bound to the CD36, HABP-1, PECAM-1 and EPCR were stained with 20 µg/ml of rabbit polyclonal antibodies against the NTS.DBLα of IT4var19, HB3var03 or IT4var07 and detected with Alexa 488 goat anti rabbit (green). The nuclei of the parasites were stained with 1 µg/ml DAPI (blue). Alexa488 images of the IEs are placed next to the DAPI images of the same IEs.

CD36

IEs from the culture of all the four parasite lines bound to CD36 (Figure 5.2), however, these CD36 bound IEs were non-DC 8 and 13 -expressing IEs (Figure 5.3) and hence likely to be the IEs expressing group B and C variants in culture (Smith *et al.*, 2013; Cabrera, Neculai and Kain, 2014). CD36 is not thought to

play a role in HBEC-binding as there is sparse or no CD36 expression on HBEC (Turner *et al.*, 1994).

PECAM-1

IEs from the 9197.HBEC cultures showed significant levels of binding to PECAM-1, and in addition to EPCR, IT4var19 IEs also bound to PECAM-1 (Figure 5.2 and Figure 5.3). Although the HB3var03 is a DC13 PfEMP1, the same PfEMP1 can also be classified as DC5 due to the DBLγ12-DBLδ5-CIDRβ3-DBLβ7 towards the C-terminal (Rask *et al.*, 2010). DC5-expressing IEs have been previously reported to bind to PECAM-1 (Berger *et al.*, 2013), however, the HB3var03 IEs did not bind to PECAM-1.

NCAM-1 and hyaluronic acid

IEs from the IT4var07 culture also bound significantly to NCAM-1 and hyaluronic acid (not significantly), however, the binding could not be subsequently reproduced for an IFA. It is possible that the binding observed earlier was due to an NCAM-1 or hyaluronic acid binding -PfEMP1 being expressed at that time and that the parasites had switched to express other variants that do not bind NCAM-1 or hyaluronic acid.

HABP-1 and other receptors

Some binding to HABP-1 was seen in all four parasite lines, however, this was not statistically significant (Figure 5.2). Low levels of binding were also seen to other receptors including CSA and ICAM-1 but consistent and statistically significant binding were not observed. An IFA was attempted on all IEs bound to protein, however, with the exception of IEs bound to HABP-1, the IEs bound to the rest were lost at the end of the IFA. This may have been due to a relatively weaker binding affinity of the IEs to those receptors. All IEs (from IT4var07, IT4var19 and HB3var03) bound to HABP-1, expressed the DC8 or 13 PfEMP1 (Figure 5.3).

5.5.2 Only IT4var 19 IEs show EPCR-dependent adhesion to HBEC-5i

Following on from the receptor adhesion results, EPCR, CD36, HABP-1, and PECAM-1 recombinant proteins were tested for ability to block binding to HBEC. EPCR significantly inhibited (>80%) cytoadhesion of IT4var19 IEs to HBEC-5i and had no effect on adhesion of the DC13-expressing HB3var03 and IT4var07 (Figure 5.4). Similar inhibition was also seen in binding to primary cells, HBMEC (Figure 5.5).

PECAM-1 also significantly prevented adhesion of IT4var19 to HBEC-5i but had no effect on cytoadhesion of HB3var03 IEs to HBEC-5i, in agreement with the adhesion to recombinant protein result. The HABP-1 recombinant protein also had no significant effect on adhesion of all the three parasite lines; although it inhibited adhesion of IT4var19, this was not statistically significant. As expected, CD36 recombinant protein had no effect on HBEC-binding in all three parasite lines.

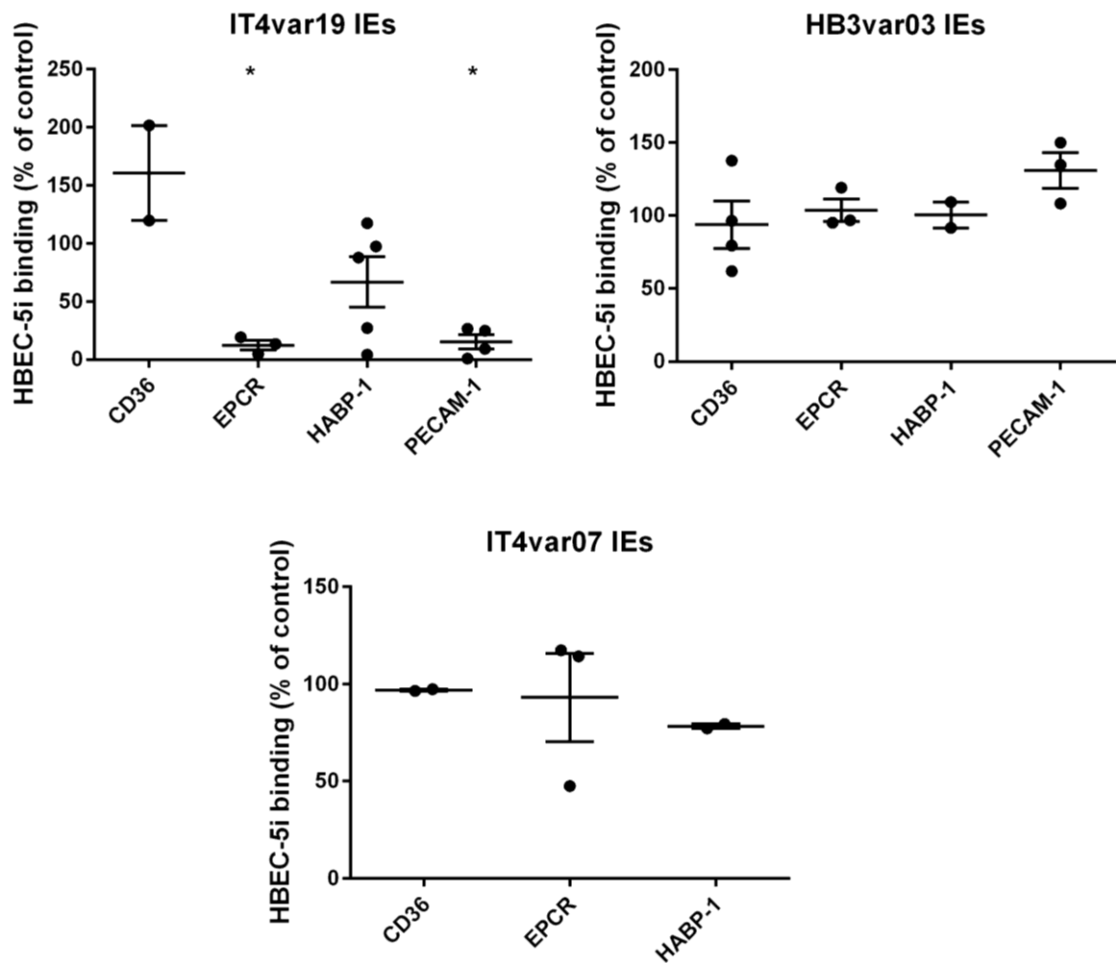


Figure 5.4. Effect of CD36, EPCR, HABP-1 and PECAM-1 recombinant proteins on adhesion of IT4var19, HB3var03 and IT4var07 IEs to HBEC-5i. Gelatin purified IEs, of at least 30% parasitaemia, were pre-incubated with 20 µg/ml of the recombinant proteins before co-incubation with HBEC-5i in a static binding assay. Each experiment included duplicate wells, and IEs bound per 100 endothelial cells were counted at 400X. The number of bound IE were compared to the control of IEs bound to HBEC in 0.1% BSA/DMEM binding medium only. Control levels varied between 181 to 345 for IT4var19 IEs, 312 to 1029 for HB3var03 IEs and 310 to 367 for IT4var07 IEs The data shown are mean and SEM of at least two independent experiments. The means were compared to that of the controls (binding in 0.1% BSA/DMEM binding medium) using one-way ANOVA with Dunnett's multiple comparisons test, $n \geq 2$; * $P < 0.05$.

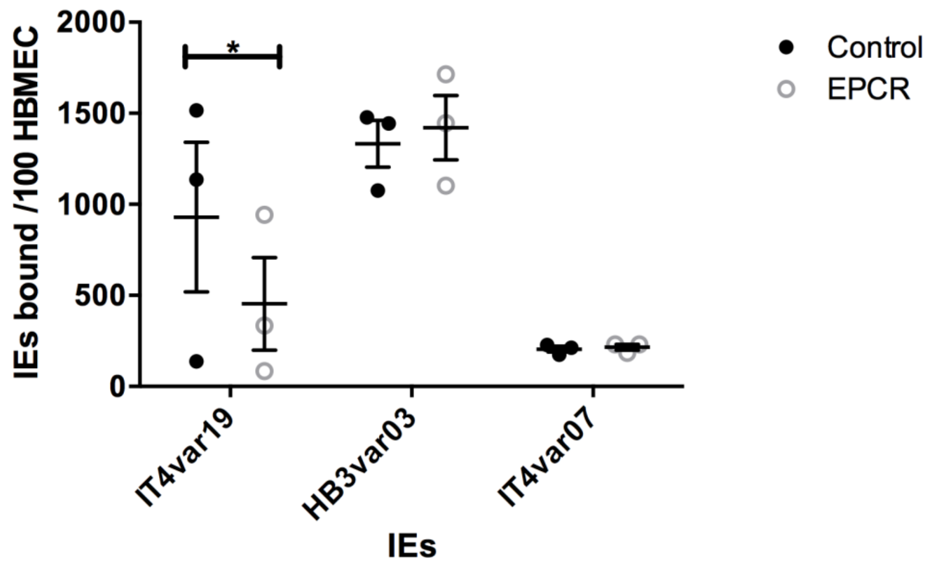


Figure 5.5. Effect of EPCR recombinant proteins on adhesion of IT4var19, HB3var03 and IT4var07 IEs to HBMEC. Gelatin purified IEs, of parasitaemia not less than 30%, were pre-incubated with 20 µg/ml of the recombinant proteins before co-incubation with HBMEC in a static binding assay. Each experiment included duplicate wells, and IEs bound per 100 endothelial cells were counted at 400X. The data shown are mean and SEM of three experiments. The means were compared in a paired t test, n = 3; * $P < 0.05$.

Antibodies to EPCR were also tested in an adhesion inhibition assay with all the four parasite lines to HBEC-5i. EPCR monoclonal and polyclonal antibodies significantly reduced binding of IT4var19 IEs to HBEC-5i ($p<0.0001$) but had no effect on adhesion of HB3var03, IT4var07 and 9197.HBEC IEs to HBEC-5i, $p>0.05$, (Figure 5.6).

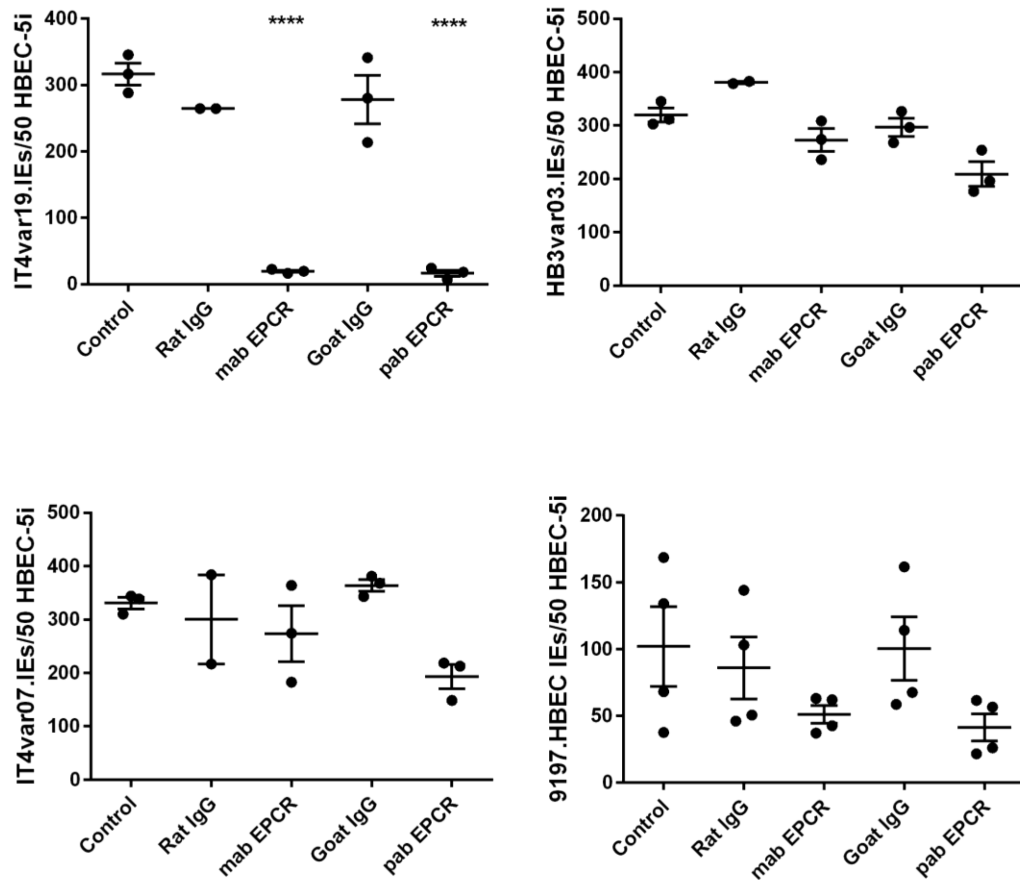
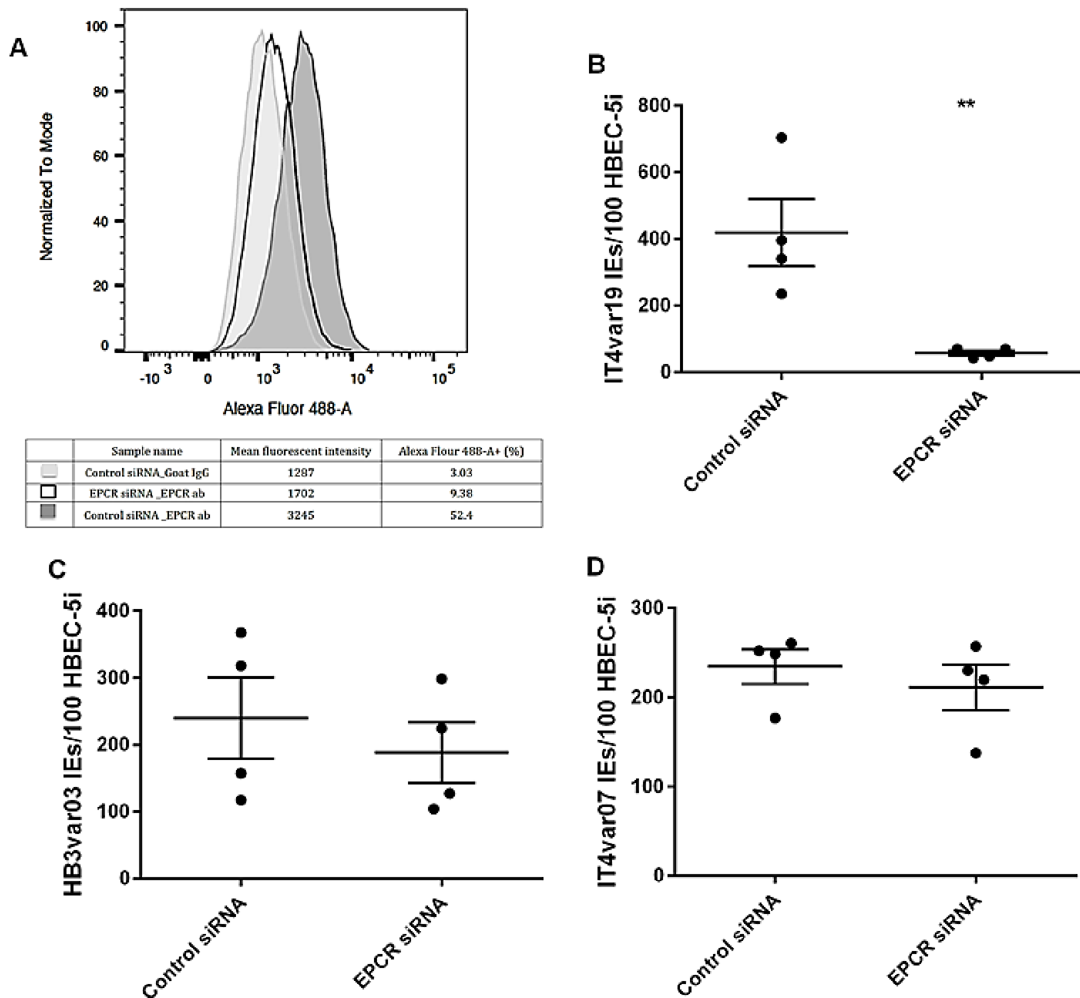


Figure 5.6. Effect of EPCR antibodies on adhesion of IT4var19, HB3var03, IT4var07 and 9197.HBEC IEs to HBEC-5i. Confluent HBEC-5i were pre-incubated with 20 µg/ml of EPCR monoclonal (mab EPCR) or polyclonal antibodies (pab EPCR), or antibody isotype controls before co-incubation with gelatin purified IEs (parasitaemia not less than 30%) in a static binding assay. Each experiment included duplicate wells, and IEs bound per 50 endothelial cells were counted at 400X. The data shown are mean and SEM of at least two independent experiments. The means were compared to that of the 'no-antibody' controls using one-way ANOVA with Dunnett's multiple comparisons test, $n \geq 2$; **** $P < 0.0001$.

5.5.3 Knockdown of EPCR expression on HBEC-5i only inhibits cytoadhesion of IT4var19 IEs

To further examine the role of EPCR in cytoadhesion of these parasite lines to HBEC-5i, the expression of EPCR on HBEC-5i was silenced with EPCR siRNA and binding of the IEs to the HBEC-EPCR knock down tested (HBEC-EPCRkd). The expression of EPCR on the surface on the HBEC-5i was reduced by >50% 48 hours after transfection (Figure 5.8). The transfected cells were also stained with ICAM-1 antibodies to ensure that the transfection had no effect on other proteins expressed on the cells. Compared to controls, which were HBEC-5i transfected with control siRNA A (a scrambled sequence with no effect on expression), there was no significant difference in binding of IT4var07 and HB3var03 IEs to HBEC-EPCRkd however IT4var19 IEs binding was reduced to 15.2% (Figure 5.7). This shows that while EPCR mediates the binding of IT4var19 to HBEC-5i, it is not involved in HBEC-binding of HB3var03 and IT4var07.



	Sample name	Mean fluorescent intensity	Alexa Flour 488-A+ (%)
	Control siRNA_Goat IgG	1287	3.03
	EPCR siRNA _EPCR ab	1702	9.38
	Control siRNA _EPCR ab	3245	52.4

Figure 5.7. EPCR knockdown only affects adhesion of IT4var19 to HBEC-5i. A. Expression of EPCR on HBEC-5i under flow cytometry. HBEC-5i was transfected 30 nmol EPCR siRNA or mock transfected with 30 nmol of a scramble sequence, control A siRNA. Flow cytometry was done on 2% PFA-fixed cells, 48 hours after transfection. The cells were stained with 1 µg/ml goat polyclonal antibody to human EPCR in 1.25 µg/ml Hoechst/PBS, washed and stained with 1 in 1500 Alexa Fluor 488 donkey in 1.25 µg/ml Hoechst/PBS. Goat IgG was used for the isotype control. B – D. Static binding of gelatin purified IEs to HBEC-control siRNA and HBEC-EPCR-siRNA transfected cells. Data shown are mean and SEM of four independent experiments, n = 4, each with duplicate wells. The means were compared in a paired t test; ** P<0.01

5.5.4 CIDRα1.1 and 1.4 recombinant proteins inhibit binding of IT4var19 IEs to EPCR

The CIDRα1.1 and CIDRα1.4 domains have been suggested to be the HBEC binding domain of the DC8 and DC13 PfEMP1 (Turner *et al.*, 2013). CIDRα1.1 and 1.4 recombinant proteins from IT4var19, HB3var03 and IT4var07 PfEMP1 were shown to bind to EPCR with high affinities, with Kd values of 16 nM, 0.37 nM and 1.3 nM for IT4var19, HB3var03 and IT4var07 respectively (Turner *et al.*, 2013). The crystal structure of IT4var7 and HB3var03 CIDRα1.4 recombinant proteins bound to EPCR was solved and the binding site on EPCR shown to overlap with that of protein C (Lau *et al.*, 2015). However, I have shown in Figure 5.1 to Figure 5.7 that only IT4var19 IEs but not the others, IT4var07 and HB3var03 IEs actually bind to EPCR.

To investigate the conflicting results, the CIDRα1 of the parasites were spotted onto EPCR already absorbed onto a dish and IT4var19 IEs added to the dish. CIDRα1 of all the three parasite lines blocked binding of IT4var19 IEs to EPCR (Figure 5.8). Binding of CD36 expressing variants in the culture was not inhibited by the CIDRα1 recombinant proteins. This result suggests that all three CIDRα1a recombinant proteins bind to EPCR, although the DC13-expressing IEs have been shown not to bind to EPCR.

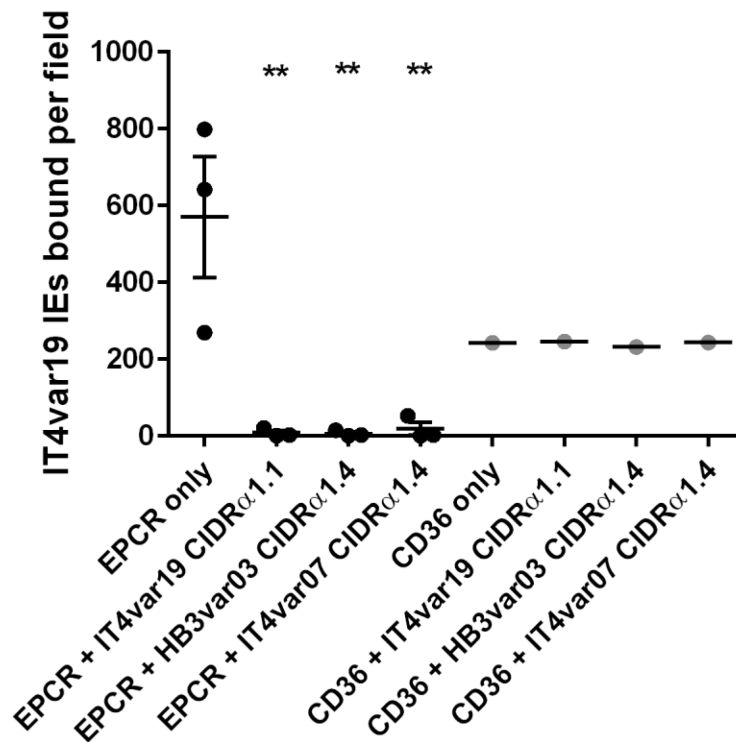


Figure 5.8. CIDRα 1.1 and 1.4 recombinant proteins inhibit binding of IT4var19 IEs to EPCR. Recombinant proteins, CIDRα1.1 of IT4var19 and CIDRα1.4 of IT4var07 and HB3var03 were added to the spots of EPCR or CD36 recombinant proteins adsorbed onto a dish for 30 minutes before gelatin purified IT4var19 IEs were added for binding. Each experiment included duplicate dishes, each with two spots per proteins and IEs were counted for three fields per spot at 400X. Data shown are mean and SEM of three independent experiments, n = 3 with EPCR proteins and the data of one experiment with CD36 protein. The means were compared to their respective EPCR or CD36 only control using one-way ANOVA with Dunnett's multiple comparisons test; ** $P < 0.01$.

5.5.5 Serum inhibition of cytoadhesion of IT4var19 to HBEC-5i is independent of EPCR

Human serum contains soluble EPCR (sEPCR), with the levels ranging from 28 to 470 ng/ml in plasma in healthy individuals (Shabani *et al.*, 2016). In section 3.5.1.5 of chapter 3 Figure 3.9, human serum at 10% (v/v) was shown to abolish adhesion of IT4var19 IEs to HBEC-5i; furthermore, EPCR at a concentration of

20 µg/ml, was shown to reduce adhesion of IT4var19 IEs to HBEC-5i by more than 80% (Figure 5.4). Although the level of sEPCR in the DMEM binding medium with serum, was not determined, it was possible that sEPCR in the serum inhibited the binding of IT4var19 IEs to HBEC-5i.

To test this hypothesis, serum was depleted of EPCR with EPCR antibody coated beads and the depletion confirmed by ELISA (Table 5.1).

Sample	EPCR Concentration (ng/ml)
Human serum	57.41
EPCR depleted human serum	0
CD36	0

Table 5.1. EPCR concentration in normal serum and EPCR-depleted serum. A sandwich ELISA was done with 1.0 µg/ml EPCR monoclonal antibody coated plates. Recombinant human EPCR at 0.31, 0.63, 1.25, 2.50 and 5.00 ng/ml (for the calibration curve) and, human serum and the EPCR-depleted human serum diluted 1 in 10 in blocking buffer were added to the coated plate together with CD36 at 5 ng/ml (a negative control). EPCR goat polyclonal antibody at 0.5 µg/ml was used as the primary detection antibody and 1/3000 HRP- anti-goat IgG used as the secondary antibody. OPD (0.04 mg/ml and 0.012% v/v H_2O_2 was used to develop the reaction which was stopped with 12 M H_2S0_4 after 15 minutes. The optical density per well was read at 492 nm. Presented in the table are the extrapolated values (with dilution accounted for) from the calibration curve that was generated.

EPCR depleted serum inhibited IT4var19 IEs binding to HBEC-5i to a similar extent as the normal human serum (Figure 5.9). Therefore, there is an unknown human serum protein or factor inhibiting IE adhesion to EPCR suggesting that binding to EPCR may not be physiologically relevant.

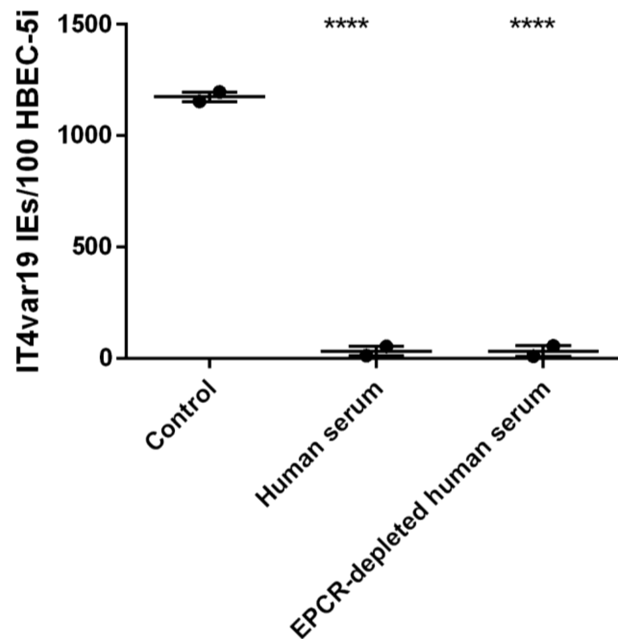


Figure 5.9. EPCR-depleted human serum abolishes IT4var19 IEs adhesion to HBEC-5i adhesion. Human serum was depleted of EPCR using EPCR polyclonal antibody coated Dyna beads. Human serum or EPCR-depleted serum was added to gelatin purified IEs (parasitaemia of at least 30%) at 10% concentration in DMEM binding medium and co-incubated with HBEC-5i. The data shown are mean and SEM of two independent experiments; n = 2. The means were compared to that of the controls using one-way ANOVA with Dunnett's multiple comparisons test; **** $P < 0.0001$.

5.6 Discussion

It is shown, in this chapter, that EPCR is not the receptor for adhesion of DC13-expressing IEs to HBEC. The three parasite lines used, HB3var03, IT4var07 and IT4var19 IEs all bind avidly to HBEC-5i but the EPCR knockdown, protein and antibodies experiments show that while EPCR appears to mediates adhesion of IT4var19 IEs to brain endothelial cells, as reported by others (Avril *et al.*, 2013; Turner *et al.*, 2013), the cytoadhesion of the other two HB3var03, IT4var07 parasite lines, is independent of EPCR.

Differences in cytoadhesion and the role of EPCR in adhesion of IT4var07 and IT4var19 IEs to HBMEC and transformed HBMEC (THBMEC) have been reported. IT4var07 IEs were reported not to bind to primary HBMEC but to bind to THBMEC and cytoadhesion was inhibited (33%) by EPCR (Gillrie *et al.*, 2015). The study also reported that binding of IT4var19 IEs to primary HBMEC was not mediated by EPCR contrary to what was shown in Figure 5.5. In this study, IT4var07, IT4var19 and HB3var03 IEs, all bound to HBMEC and HBEC-5i, although binding of IT4var07 IEs to HBMEC appeared to be relatively low. EPCR also inhibited adhesion of only IT4var19 IEs to HBMEC and had no effect on the adhesion of IT4var07 and HB3var03 IEs (Figure 5.5), similar to that of binding to HBEC-5i. A comparative study of binding of the IEs to HBEC and THBMEC would have to be done at the same time with equal number of cells to determine the any difference in the efficiency of binding of IEs to the two cell lines.

Data presented in this chapter show a discrepancy between the CIDRα1-EPCR recombinant protein binding and the IEs-EPCR binding. Binding of IT4var19 IEs to EPCR was inhibited by the recombinant HB3var03 and IT4var07 CIDRα1.4 suggesting the CIDRα1.4 recombinant proteins bind to EPCR as have been shown by others (Turner *et al.*, 2013; Lau *et al.*, 2015), however, the CIDRα1.4-EPCR binding did not prevent the HB3var03 and IT4var07 IEs to HBEC-5i (in chapter 4, figure 4.20). Although the DC13 CIDRα1 recombinant proteins bind to EPCR, there appears to be a different receptor for HBEC-binding of the DC13-expressing IEs, and this interaction seems to occur regardless of binding to EPCR. An explanation for the differences between the CIDRα1-EPCR protein binding and the IEs-EPCR binding is that the conformation of the CIDRα1.4 recombinant proteins may be different from that of the native PfEMP1 on the IEs. Such that the EPCR binding site on the CIDRα1.4 recombinant proteins is not exposed on the IEs. Post-translational modifications can cause steric hindrance in a protein and affect the conformation and binding properties of the protein (Houde *et al.*, 2010). It is possible the recombinant proteins lack post-translational modifications that are on the native PfEMP1 domains expressed on the surface of the IEs.

Dual binding phenotype has been described for single 3D7 IEs (Joergensen *et al.*, 2010). These IEs were reported to express two different PfEMP1s, PFD1235w (ICAM-1 binder) and PF11_0008 (PECAM-1 binder) on a single IE and could bind to both ICAM-1 and PECAM-1 on transfected CHO and HUVEC cells. Using a novel approach of immunofluorescence staining of protein-bound IEs, it was shown that the IEs expressing IT4var19 bound to both EPCR and PECAM-1 and the recombinant proteins also inhibited adhesion to HBEC (Figure 5.4). The IT4var19 is a group B/A PfEMP1 with both group A and group B features (Avril *et al.*, 2012; Smith *et al.*, 2013). Although recombination between group A and B *var* genes are uncommon (Rask *et al.*, 2010; Claessens *et al.*, 2014), the DC8 chimeric of both group B and A may be an adaption that enables the IEs to bind to multiple receptors.

EPCR is highly expressed on endothelial cells lining large vessels and can also be found on other cells such as monocytes and smooth muscle cells (Rao, Esmon and Pendurthi, 2014) The expression of EPCR is, however, relatively low on endothelial cells lining the microvasculature of the brain than in other tissues (Laszik *et al.*, 1997) and loss of EPCR at sites of sequestration have been reported (Moxon *et al.*, 2013). Hence, IEs binding to EPCR does not explain why there is significantly more sequestration in the brain than in other organs in cerebral malaria (Milner *et al.*, 2014).

There is also soluble EPCR (sEPCR) in plasma which competes with the membrane bound EPCR for binding to protein C (Pintao *et al.*, 2011). It is therefore, expected that sEPCR in plasma might inhibit binding of the DC8 and DC13 -expressing IEs to the minimally expressed membrane bound EPCR, however, sEPCR did not account for the ability of human serum to inhibit binding of the IT4var19 IEs (Figure. 5.9). The serum inhibitory effect may be either due to a modification or blockage of the EPCR binding site on the HBEC or on the DC8 PfEMP1 expressed by the IEs. The IEs used in this study were cultured in human serum it is, therefore, unlikely the serum inhibitory factor may act by modifying the PfEMP1 expressed on the surface of the IEs.

EPCR may be essential in the pathology of severe malaria as there is loss of EPCR at the sites of sequestration in the brain (Moxon *et al.*, 2013). This

phenomenon, however, may not be due to the direct binding of IEs to EPCR, as the EPCR is rather shed or lost at those sites. The cytokines, TNFα and IL-1β (Armah *et al.*, 2005) which are associated with cerebral malaria (increased expression), have been shown to reduce the mRNA and surface protein expression of EPCR on HUVEC and HBEC, and increase shedding of sEPCR (Menschikowski *et al.*, 2009; Gillrie *et al.*, 2015). This may fit with increase in levels of plasma sEPCR associated with cerebral malaria (Moussiliou *et al.*, 2014) and the association of the rs867186 (Ser219Gly) genotype, a non-synonymous single nucleotide polymorphism (SNP) of the PROCR gene (EPCR encoding gene) which causes expression of high levels of sEPCR, with protection from severe malaria in adults and children (Naka *et al.*, 2014; Shabani *et al.*, 2016). Other studies, however, reported no genetic variability in the PROCR gene associated with cerebral or severe malaria in children (Schuldt *et al.*, 2014; Hansson *et al.*, 2015).

This study suggests that although EPCR may be associated with severe malaria it may not be involved in sequestration in cerebral malaria. The study shows that a receptor (yet to be identified) other than EPCR mediates adhesion of IEs to brain endothelial cells and raises questions about whether recombinant protein-protein interactions necessarily translate to whole cell-cell or cell-protein interactions. It also calls into question, the physiological relevance of EPCR-binding in sequestration. Further studies are required to investigate the inhibitory effect of serum on EPCR-binding. More studies are required with other laboratory and clinical isolates to confirm the differences in cytoadhesion of DC8 and DC13 -expressing parasites and identify the receptor mediating adhesion to HBEC. HABP-1 may be a receptor worth exploring as it was bound by all the HBEC-binding parasites even though the binding was not statistically significant.

6 Chapter VI: Conclusion

Binding of IEs to brain endothelial cells contributes to sequestration of IEs in the brain microvasculature in individuals with cerebral malaria. Current treatment of the disease is with parasite-killing anti-malarial drugs but the mortality rates are still high (15% - 20%) (Miller *et al.*, 2013), and continued cytoadherence of dead parasites after anti-malarial treatment has been shown (Hughes, Biagini and Craig, 2010). Knowledge of this binding interaction is therefore essential for the development of adjunctive therapies that target the fundamental pathology of IEs sequestration to supplement existing drugs, as recent adjunctive therapies targeted at the other pathophysiological processes associated with cerebral malaria have either failed or are yet to prove beneficial (Krishna, 2012).

The aim of this thesis was to understand the mechanisms involved in the binding of IEs to brain endothelial cells by investigating the environmental conditions optimal for binding, the parasite ligand involved and the receptor on the brain endothelial cells. This chapter seeks to provide details of how this study contributes to current knowledge of parasite binding interaction with brain endothelial cells, limitations of the study and recommendations for future studies.

6.1 The parasite and HBEC binding interaction

Changes in environmental conditions in the microvasculature such as pH, temperature and gas can affect the endothelial glycocalyx which consists of charged glycolipids, glycoproteins and proteoglycans that may be involved in cytoadhesion, and can also cause changes in the binding ligand expressed on the surface of the HBEC-binding IEs. For the first time, this study shows how these environmental conditions can affect binding of the DC8 and DC13 -expressing IEs to HBEC.

In chapter three, it is shown that differences in oxygen concentrations (hypoxia and normoxia) do not affect binding of IEs to HBEC. The data suggest

hypoxia, which is associated with cerebral malaria (White *et al.*, 1985), may have no direct effect on the receptor and ligand involved in the binding. For experimental reasons, adhesion of IEs to HBEC in high CO_2 (5%) was compared to low CO_2 (0.03%) in air and found to be optimal in 5% CO_2, which is used in HBEC culture for maintaining the pH (7.3) of the $NaHCO_3$-buffered DMEM medium. However, $NaHCO_3$-free medium that had HEPES only as the buffer (DMEM binding medium) was found to resist pH changes when exposed to air containing 0.03% CO_2 and is therefore recommended for binding assays as no significant differences was observed in binding in both media under the respective gaseous conditions.

Metabolic acidosis and its related respiratory distress which are both features of cerebral malaria are the strongest predictors of fatal outcome of malaria (Marsh *et al.*, 1995; Maitland and Marsh, 2004; Warimwe *et al.*, 2012). Blood pH of individuals with cerebral malaria can range from 7.16 to 7.36 (English *et al.*, 1997). Adhesion of all the parasite lines to HBEC-5i was optimal at pH 7.3 with no significant difference at pH 7.2. This data suggest that blood pH range associated with metabolic acidosis in individuals with cerebral malaria, is conducive for binding of IEs to HBEC, and also gives the optimal pH for HBEC-binding assays.

Fever, which is characterized by high temperature, is associated with malaria and attributed to the rupture of schizont IEs and hence synchronicity of the parasites (Kwiatkowski, 1989). There has been no correlation of febrile temperatures to outcome of the disease (Akpede, Sykes and Abiodun, 1993; Farnert *et al.*, 1997; Delley *et al.*, 2000; Esamai *et al.*, 2001; Mayor *et al.*, 2011). It was therefore not surprising that there was no effect on adhesion at a high temperature of 39°C. However, increase in expression of PfEMP1 at febrile temperatures of 40°C have been shown to increase cytoadhesion of IEs to CD36 or ICAM-1 (Udomsangpetch *et al.*, 2002). Differences in the data may be due to their exposure of the IEs to higher temperatures of 40°C and the receptors the IEs bind to.

Adhesion of IEs to HBEC-5i was found to increase with increase in parasitaemia. This was in agreement with previous studies with melanoma cells

(Marsh *et al.*, 1988; Ho and Singh, 1991). IEs that bind to endothelial cells have been reported to promote subsequent binding of IEs to endothelial cells by stimulating an increase in expression of adhesion receptors such as ICAM-1 (Viebig *et al.*, 2005). This may be worth exploring to see if an increase in receptors may occur with binding of the DC8 and DC13 -expressing IEs to HBEC, although EPCR has rather been shown to be lost at sites of sequestration (Moxon *et al.*, 2013). Most importantly, for experiments, this data shows the need to control for differences in parasitaemia for comparative adhesion assays involving different isolates or parasite lines. It should be noted that the IEs used were all gelatin purified for IEs with knobs which is essential for binding to HBEC (Udeinya *et al.*, 1981; Claessens *et al.*, 2012 and section 4.5.1) and not accounting for knobs in the parasitaemia may not necessarily give the same result.

A major finding of this study is how crucial it is to include serum in the selection of HBEC-binding IEs and in the IE adhesion to HBEC assays. It was shown that human serum (from malaria-naive individuals) blocked binding (>95% inhibition) of the DC8-expressing parasite line to HBEC. This suggests that, the DC8-expressing parasite line may not bind to HBEC under physiological conditions. The human serum inhibitory effect was, however, not enough (<45%) to prevent the adhesion of DC13-expressing parasite lines to HBEC. Human serum may provide some form of protection, in the form of cytokines or serum factors, against sequestration that may regulate adhesion of the DC13 PfEMP1-expressing parasite lines but prevent adhesion of the DC8-expressing parasite lines. Only one DC8-expressing parasite line, IT4var19, was tested, therefore this results needs to be confirmed using more DC8 and DC13 - expressing parasite lines, and the mechanism of the serum inhibition needs to be investigated.

This study gives an insight into how environmental conditions can affect the adhesion of the HBEC-binding IEs. A caveat, however, is that in individuals with cerebral malaria, these conditions tested may not act in isolation but may occur at the same time and may be affected by interactions with cytokines and other mediators of inflammation, and other immune cells such as monocytes

associated with sequestration in cerebral malaria (Moxon *et al.*, 2013, 2014; Milner *et al.*, 2014). The possibility of effects of co-infections (which is common in sub-Saharan Africa where the disease is prevalent (Hartgers and Yazdanbakhsh, 2006; Hochman and Kim, 2009; Hochman *et al.*, 2013)) on the adhesion to HBEC-5i can also not be ruled out. In the absence of an appropriate *in vivo* model for sequestration of IEs in cerebral malaria (Craig *et al.*, 2012), this *in vitro* model of IEs binding to HBEC provides a good model for sequestration in cerebral malaria. The *in vitro* assay could, however, be modified for optimized and relevant binding by including the use of gelatin purified trophozoites in DMEM binding medium, supplemented with 10% human serum, at pH 7.3 for co-incubation with HBEC for an hour at 37 °C.

6.2 The parasite binding ligand

PfEMP1 is a variant surface antigen and the identification of the specific types or groups that mediate adhesion to HBEC could enable understanding of the molecular mechanism involved. The two studies that examined genes that were up-regulated in three parasite lines selected for binding to HBEC, identified the group B/A or A *var* genes that transcribe DC8 and DC13 PfEMP1 respectively (Avril *et al.*, 2012; Claessens *et al.*, 2012). PfEMP1 is a large protein (approximately 240-350 kDa) made up of combinations of DBL and CIDR domains, some of which have been shown to bind to host receptors (Kraemer and Smith, 2006). It is possible that these DC8 and DC13 PfEMP1 may share a domain (s) that mediates binding to HBEC.

6.2.1 Are the DC8 and DC13 PfEMP1 encoding *var* genes transcribed by all IEs that bind to HBEC?

Two isolates (8211 and 9197), recently adapted to culture were selected for binding to HBEC and the *var* genes they predominantly transcribed were described in chapter 4. In addition to a DC8 PfEMP1 encoding *var* gene, another subset of group A *var* genes was up-regulated after selection of the two parasite

lines for binding to HBEC and this subset was not DC13 PfEMP1 encoding *var* genes. The 9197var15 which was predominantly expressed by 9197.HBEC was not a DC8 or DC13 variant and could not be associated with the domain cassettes that have been described so far.

Based on the 5 parasite lines that have been so far selected for HBEC binding, the 11 PfEMP1 they predominantly express are all encoded by group B/A or A *var* genes (Figure 6.1). The first two domains towards the N-terminal of the 11 PfEMP1 are similar while the domains toward the C-terminal vary greatly (with the exception of second and subsequent domains of 8211 PfEMP1 'tags' which could not be determined). Parasite expression of DBLα1.2/1.7/2–CIDRα1 domains at the N-terminal may be required for binding of IEs to HBEC.

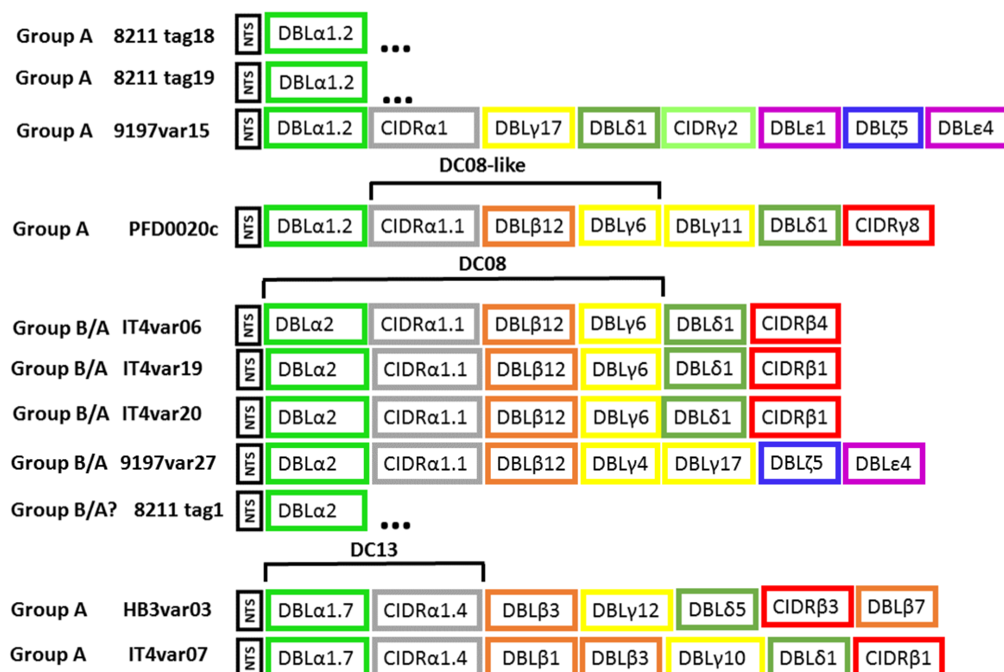


Figure 6.1. Domain architecture of PfEMP1 predominantly expressed by IEs that bind to HBEC. PfEMP1 encoded by *var* genes highly transcribed in 9197 and 8211 IEs selected for HBEC-binding in this study, and HB3 and IT4 IEs selected for HBEC-binding in three other studies (Avril *et al.*, 2012; Claessens *et al.*, 2012; Turner *et al.*, 2013). '...' in the diagram means subsequent domains of the PfEMP1 that could not be identified.

Antibodies to the NTS.DBLα of two DC13-expressing parasite lines, HB3var03, IT4var07 and the DC8-expressing parasite line, IT4var19, inhibited adhesion of the IEs to HBEC. The CIDRα1 recombinant proteins also inhibited adhesion of the IT4var19 IEs to HBEC but had no effect on cytoadhesion of the DC13-expressing parasite lines. It is possible that like the var2CSA that binds to syncytiotrophoblasts, many domains may be involved in the binding of the IEs to HBEC (Fried and Duffy, 2015) and the binding domains may also be specific to the type of PfEMP1. Although the NTS.DBLα may not necessarily be the domain of the PfEMP1 binding to HBEC-5i, it would be worth investigating, to identify any inhibition across heterologous strains (e.g. determine whether DC8 NTS.DBLα antibodies could inhibit binding of DC13-expressing IEs to HBEC), for its potential use as a therapy against IEs adhesion to HBEC in cerebral malaria.

Other parasite proteins or VSA including rifins which have been associated with rosetting (Goel *et al.*, 2015) and also found (*rif* adjacent to the upregulated group A-like *var* genes) to be upregulated on selection of IEs for adhesion to HBEC (Claessens *et al.*, 2012), need to be investigated to determine whether they are also involved in cytoadhesion to HBEC.

6.2.2 Are *var* genes transcribed by HBEC-binding IEs relevant in cerebral malaria?

Antibodies from severe malaria convalescent patients, compared with those from the patients at the time of admission or patients with uncomplicated malaria, recognise the surface of parasites that express the group A-like PfEMP1 (Jensen *et al.*, 2004; Warimwe *et al.*, 2009, 2012). A similar result was observed with plasma from patients recovering from cerebral malaria, which recognised the HBEC-binding parasites (Claessens *et al.*, 2012). This suggests that the expression of those variants may have clinical relevance.

Parasites from children who had cerebral malaria compared to those with uncomplicated malaria predominantly transcribed these DC8 and DC13 variants (Bertin *et al.*, 2013; Almelli *et al.*, 2014b). These variants are, however, also highly transcribed by parasites from children with other forms of severe

malaria (Lavstsen *et al.*, 2012). The DC8 and DC13 PfEMP1 expressed by the HBEC-binding parasites are associated with cerebral malaria but may contribute to the disease in different ways. In chapter 3, serum was shown to prevent binding of the DC8-expressing parasite line, which suggests that *in vivo* such parasites may be contributing to severe malaria through mechanisms other than binding to HBEC. In the one study that had other forms of severe malaria and cerebral malaria, high transcripts of DC13 variants were seen in the cerebral malaria patients compared to other forms of severe malaria but this was not statistically significant (Lavstsen *et al.*, 2012).

Two methods are widely used for the identification of *var* genes. The use of the DBLα universal primers that targets highly conserved sequences in all *var* genes (Bull *et al.*, 2005) enables the determination of DBLα tags (and hence the group of *var* genes transcribed). The other method involves the use of 42 primer pairs that target different DBL and CIDR domain transcript levels by real time-qPCR and enables the identification of groups of PfEMP1 and domain cassettes. From the 42 primers that have been used, there are no specific primers that target the DBLα 1.2 or 1.7 only, however, there are primers for 'DBLα2/1.2/1.4/1.7' combined, 'DBLα not var3' or DBLα_CIDRα (classified as DC8) and transcripts of all of those domains were significantly higher in cerebral malaria compared to uncomplicated malaria (Lavstsen *et al.*, 2012); although the other studies which tested the 'DBLα2/1.2/1.4/1.7' or 'DBLα_CIDRα' did not report any such differences (Bertin *et al.*, 2013; Almelli *et al.*, 2014b). Some of the CIDRα1 domains correlated with cerebral malaria, however, there were differences in the types that were significantly transcribed in cerebral malaria patients between the studies (Lavstsen *et al.*, 2012; Bertin *et al.*, 2013; Almelli *et al.*, 2014b; Bernabeu *et al.*, 2016; Jespersen *et al.*, 2016). The association of parasite expression of DBLα1.2/1.7-CIDRα1 with HBEC-binding and cerebral malaria needs to be investigated using cerebral malaria isolates, with controls from other forms of severe malaria and uncomplicated malaria, to test the hypothesis that parasites from cerebral malaria patients bind to HBEC and would predominantly transcribe the DBLα1.2/1.7-CIDRα1 encoding *var* genes.

6.3 The HBEC receptor

P. falciparum have been shown to bind to several host cell receptors and the expression of certain PfEMP1 types have been linked to these receptors. However, with the exception of adhesion of IEs to CSA in PAM, association of binding to receptors, to type of disease have so far been inconclusive. For cerebral malaria the implicated receptors are ICAM-1 and EPCR, however, for ICAM-1, there is conflicting data about association of binding to ICAM-1-with cerebral malaria (reviewed in Craig, Khairul and Patil, 2012) (Nogueira *et al.*, 2002; Almelli *et al.*, 2014b). Relatively few studies have investigated any correlation between binding to EPCR and cerebral malaria.

6.3.1 Do all HBEC-binding IEs bind to EPCR on HBEC?

IEs that bind to HBEC have been described to bind to EPCR. These parasites predominantly express DC8 and DC13 PfEMP1, and the DC8 and DC13 PfEMP1 recombinant proteins bind with high affinity to EPCR, a receptor on HBEC (Turner *et al.*, 2013; Lau *et al.*, 2015), it can, therefore, be concluded that EPCR is the receptor for binding of IEs to HBEC. This was, however, not the case for all the HBEC-binding IEs used in this study. In chapter 5, it was shown in detail that HBEC-binding IEs that predominantly express DC13 PfEMP1 do not bind to EPCR on HBEC. The DC8-expressing parasite line on the other hand binds to EPCR on HBEC.

The DC13 PfEMP1 CIDRα1 domain recombinant proteins inhibited binding of the DC8-expressing parasite line to HBEC but had no effect on adhesion of the DC13-expressing parasite lines to HBEC. This may indirectly confirm that these CIDRα1 recombinant proteins bind to EPCR to prevent binding of the DC8-expressing parasite line to EPCR on HBEC but the CIDRα1-EPCR interaction is not involved in HBEC adhesion of the DC13-expressing parasite lines. Recombinant proteins of other CIDRα1 variants which are not associated with binding to HBEC, including CIDRα1.6 (part of DC4 PfEMP1, an

ICAM-1 binding variant (Bengtsson *et al.*, 2013)) have also been shown to bind to EPCR (Lau *et al.*, 2015). These results suggest that with the exception of the DC8 CIDRα1, the recombinant proteins (DC13 CIDR α1) may not be in the same conformation as that of the domain in the native PfEMP1 on the surface of the IEs to facilitate binding of the IEs to EPCR on HBEC, and shows that EPCR is not the receptor for the DC13-expressing parasite lines.

6.3.2 Is parasite binding to EPCR associated with cerebral malaria?

Binding of IEs to EPCR is suggested to contribute to cerebral malaria through the inhibition of Protein C binding to EPCR (Turner *et al.*, 2013; Gillrie *et al.*, 2015; Lau *et al.*, 2015; Sampath *et al.*, 2015). Protein C is activated through binding to EPCR and the activated Protein C (APC) has anti-coagulative and cytoprotective effects in endothelial cells (Rao, Esmon and Pendurthi, 2014). Therefore, the loss of these effects due to IE binding to EPCR is suggested to contribute to the pathology of cerebral malaria (Mosnier and Lavstsen, 2016). Binding of DC8-expressing IEs to HBEC, however, did not inhibit the generation of APC nor affect permeability of HBEC (Gillrie *et al.*, 2015).

Binding of clinical isolates from severe malaria patients to HBEC has been shown to be significantly inhibited by EPCR, in comparison to isolates from uncomplicated malaria patients (Turner *et al.*, 2013). These severe malaria isolates included isolates from cerebral malaria and severe anaemia cases. As mentioned above in section 6.2, the DC8-expressing IEs that bind to EPCR are associated with severe malaria but are not specific to cerebral malaria only.

EPCR is encoded by the PROCR gene and a single nucleotide change from serine to glycine in the exon 4 results in the rs867186-G variant which causes increased shedding of the transmembrane EPCR into plasma (Medina *et al.*, 2014). Conflicting data has been reported regarding association of the rs867186-G variant with severe malaria (Moussiliou *et al.*, 2014; Naka *et al.*, 2014; Schuldt *et al.*, 2014; Hansson *et al.*, 2015; Shabani *et al.*, 2016), and those that found a protective association with severe malaria did not observe any

differences between cerebral malaria and other forms of severe malaria (Naka *et al.*, 2014; Shabani *et al.*, 2016).

EPCR and DC8-expressing IEs may be involved in the pathology of severe malaria but this mechanism may not be due to HBEC-binding in cerebral malaria, as it has also been shown in chapter 3 that the DC8-expressing IEs do not bind to HBEC in the presence of serum and therefore binding to EPCR on HBEC may not be physiologically relevant.

6.3.3 Other receptors that could mediate adhesion to HBEC

From the ten receptor molecules that were tested in chapter 5, only binding to HABP-1 (also known as gC1qR or p32) was observed in all the DC8 and DC13 -expressing parasite lines tested, even though the binding was minimal. Significant binding of HBEC-binding IT4 IEs (predominately expressing both DC8 and DC13 PfEMP1) to HABP-1 has been reported (Claessens *et al.*, 2012), although as in this study, no significant binding was seen with the HB3var03 IEs or IT4var19 IEs only (Avril *et al.*, 2012; Claessens *et al.*, 2012). Binding of IEs to HABP-1 on HBEC had been previously reported (Biswas *et al.*, 2007) and IEs from children who had severe malaria with multiple seizures compared to uncomplicated malaria isolates have been shown to bind to HABP-1 (Mayor *et al.*, 2011).

HABP-1 is expressed intracellularly and on the surface of HBEC. HABP-1, is a receptor for Factor XII (coagulation pathway) and C1q (complement system), and also involved in several other binding interactions (Ghebrehiwet *et al.*, 1994, 2006; Treutiger *et al.*, 1997; van Leeuwen and O'Hare, 2001). Adhesion of the HBEC-binding parasites to HABP-1 may play a role in cerebral malaria and should be investigated further with field isolates and more HBEC-binding parasites.

The Retrogenix screening that identified EPCR (Turner *et al.*, 2013) is the largest screening done to date with PfEMP1. The screening was done with only a DC8 PfEMP1, IT4var20, covered less than 40% of known human plasma proteins and did not include other potential receptors such as HABP-1 and

glycosaminoglycans (Turner *et al.*, 2013). Findings of this study show that the DC8 and DC13 -expressing parasite lines bind to different receptors, therefore, subsequent studies for identification of the HBEC receptor should include the DC13 PfEMP1 and other DBLα1.2/1.7-CIDRα1 that may be associated with HBEC binding. The hits should also be validated with the HBEC-binding IEs since the data from only recombinant protein interactions can be misleading, as has been shown here.

6.4 HBEC-5i - a model for cytoadherence in cerebral malaria

HBEC-5i, which is an immortalised cell line derived from microvascular cells of the cerebral cortex of a human adult provides a good model for the studying the blood brain barrier (Dorovini-Zis, Prameya and Bowman, 1991; Weksler, Romero and Couraud, 2013). HBEC-5i cells form a monolayer of adherent cells that exhibit a contact-inhibited type of growth. The cells express surface proteins von Willebrand factor, ICAM-1 and VCAM-1 (Wassmer *et al.*, 2006) and junction-associated proteins such as and zonula occludens and PECAM-1 and do not express CD36 (Claessens, 2010, PhD; Rowe *et al.*, unpublished data), characteristic of human brain endothelial cells. Low permeability and high trans-endothelial electrical resistance (TEER) values which decrease on activation with TNF have also been reported for HBEC-5i; indicative of the integrity of the tight junctions formed by the cells (Wassmer *et al.*, 2006; Weksler, Romero and Couraud, 2013).

With the limited availability of primary HBMEC cells and differences in batches of HBMEC commercially available (Bouïs *et al.*, 2001), HBEC-5i serves as an appropriate cell line for the *in vitro* study of cytoadhesion in cerebral malaria, a disease that has been associated with the breakdown of the blood brain barrier (Rénia *et al.*, 2012). Further characterisation of the HBEC-5i, which may include a comparative transcriptional analyses of HBEC-5i and isolated primary human brain endothelial cells may be useful to confirm its relevance as a model for IE binding in the brain endothelium.

Overall, this study has shown that *in vitro* binding of IEs to HBEC is pH-dependent, optimal at pH 7.2-7.3, parasitaemia-dependent and not significantly affected by febrile temperature of 39°C. The importance of the use of human serum in binding assays has been highlighted; as HBEC-binding of the DC8-expressing variant was prevented by human serum. IEs that bind to HBEC have been shown to also predominantly transcribe *var* genes that encode non- DC8 or DC13 DBLα1.2-containing PfEMP1 variants, in addition to DC8-expressing variants. Finding of this thesis lastly show that the EPCR-CIDRα1 interaction does not mediate binding of all HBEC-binding variants and that binding to EPCR may not be physiologically relevant; other receptors may be involved in the HBEC-binding of the DC13-expressing IEs.

References

Abdel-Latif, M. S., Dietz, K., Issifou, S., Kremsner, P. G. and Klinkert, M. Q. (2003) 'Antibodies to *Plasmodium falciparum* rifin proteins are associated with rapid parasite clearance and asymptomatic infections', *Infection and Immunity*, 71(11), pp. 6229–6233.

Adams, Y., Kuhnrae, P., Higgins, M. K., Ghumra, A. and Rowe, J. A. (2014) 'Rosetting *Plasmodium falciparum*-infected erythrocytes bind to human brain microvascular endothelial cells *in vitro*, demonstrating a dual adhesion phenotype mediated by distinct *P. falciparum* erythrocyte membrane protein 1 domains', *Infection and Immunity*, 82(3), pp. 949–959.

Adams, Y. and Rowe, J. A. (2013) 'The effect of anti-rosetting agents against malaria parasites under physiological flow conditions', *PLoS ONE*, 8(9).

Aidoo, M., Terlouw, D. J., Kolczak, M. S., Nahlen, B. L., Lal, A. A. and Udhayakumar, V. (2002) 'Protective effects of the sickle cell gene against malaria morbidity and mortality', *Lancet*, 359, pp. 1311–1312.

Akpede, G. O., Sykes, R. M. and Abiodun, P. O. (1993) 'Convulsions with malaria: Febrile or indicative of cerebral involvement?', *Journal of Tropical Pediatrics*, 39(6), pp. 350–355.

Almelli, T., Ndam, N. T., Ezimegnon, S., Alao, M. J., Ahouansou, C., Sagbo, G., Amoussou, A., Deloron, P. and Tahar, R. (2014b) 'Cytoadherence phenotype of *Plasmodium falciparum*-infected erythrocytes is associated with specific PfEMP-1 expression in parasites from children with cerebral malaria.', *Malaria Journal*, 13(333).

Almelli, T., Nuel, G., Bischoff, E., Aubouy, A., Elati, M., Wang, C. W., Dillies, M. A., Coppee, J. Y., Ayissi, G. N., Basco, L. K., Rogier, C., Ndam, N. T., Deloron, P. and Tahar, R. (2014a) 'Differences in gene transcriptomic pattern of *Plasmodium falciparum* in children with cerebral malaria and asymptomatic carriers', *PLoS ONE*, 9(12), p. e114401.

Angeletti, D., Sandalova, T., Wahlgren, M. and Achour, A. (2015) 'Binding of subdomains 1/2 of PfEMP1-DBL1 α to Heparan sulfate or heparin mediates *Plasmodium falciparum* rosetting', *PLoS ONE*, 10(3), p. e0118898.

Antonova, O. A., Loktionova, S. A., Romanov, Y. A., Shustova, O. N., Khachikian, M. V and Mazurov, A. V (2009) 'Activation and damage of endothelial cells upon hypoxia/reoxygenation. Effect of extracellular pH.', *Biochemistry*, 74(6), pp. 605–612.

Armah, H., Wired, E. K., Dodoo, A. K., Adjei, A. A., Tettey, Y. and Gyasi, R. (2005) 'Cytokines and adhesion molecules expression in the brain in human cerebral malaria.', *International Journal of Environmental Research and Public Health*, 2(1), pp. 123–131.

Ashley, E. A., Dhorda, M., Fairhurst, R. M., Amaratunga, C., Lim, P., Suon, S., Sreng, S., Anderson, J. M., Mao, S., Sam, B., Sopha, C., Chuor, C. M., Nguon, C., Sovannaroth, S., Pukrittayakamee, S., Jittamala, P., Chotivanich, K., Chutasmit, K., Suchatsoonthorn, C., Runcharoen, R., Hien, T. T., Thuy-Nhien, N. T., Thanh, N. V., Phu, N. H., Htut, Y., Han, K.-T., Aye, K. H., Mokuolu, O. A., Olaosebikan, R. R., Folaranmi, O. O., Mayxay, M., Khanthavong, M., Hongvanthong, B., Newton, P. N., Onyamboko, M. A., Fanello, C. I., Tshefu, A. K., Mishra, N., Valecha, N., Phyo, A. P., Nosten, F., Yi, P., Tripura, R., Borrmann, S., Bashraheil, M., Peshu, J., Faiz, M. A., Ghose, A., Hossain, M. A., Samad, R., Rahman, M. R., Hasan, M. M., Islam, A., Miotto, O., Amato, R., MacInnis, B., Stalker, J., Kwiatkowski, D. P., Bozdech, Z., Jeeyapant, A., Cheah, P. Y., Sakulthaew, T., Chalk, J., Intharabut, B., Silamut, K., Lee, S. J., Vihokhern, B., Kunasol, C., Imwong, M., Tarning, J., Taylor, W. J., Yeung, S., Woodrow, C. J., Flegg, J. A., Das, D., Smith, J., Venkatesan, M., Plowe, C. V., Stepniewska, K., Guerin, P. J., Dondorp, A. M., Day, N. P. and White, N. J. (2014) 'Spread of artemisinin resistance in *Plasmodium falciparum* malaria', *New England Journal of Medicine*, 371(5), pp. 411–423.

Avril, M., Bernabeu, M., Benjamin, M., Brazier, A. J. and Smith, J. D. (2016) 'Interaction between Endothelial Protein C Receptor and Intercellular Adhesion Molecule 1 to mediate binding of *Plasmodium falciparum*-infected erythrocytes to endothelial cells', *mBio*, 7(4), pp. e00615-16.

Avril, M., Brazier, A. J., Melcher, M., Sampath, S. and Smith, J. D. (2013) 'DC8 and DC13 *var* genes associated with severe malaria bind avidly to diverse endothelial cells', *PLoS Pathogens*, 9(6), p. e1003430.

Avril, M., Tripathi, A. K. K., Brazier, A. J. J., Andisi, C., Janes, J. H. H., Soma,

V. L. L., Sullivan, D. J., Bull, P. C., Stins, M. F. and Smith, J. D. (2012) 'A restricted subset of *var* genes mediates adherence of *Plasmodium falciparum*-infected erythrocytes to brain endothelial cells', *Proceedings of the National Academy of Sciences*, 109(26), pp. E1782–E1790.

Bachmann, A., Scholz, J. A. M., Janßen, M., Klinkert, M.-Q., Tannich, E., Bruchhaus, I. and Petter, M. (2015) 'A comparative study of the localization and membrane topology of members of the RIFIN, STEVOR and PfMC-2TM protein families in *Plasmodium falciparum*-infected erythrocytes.', *Malaria Journal*, 14(274).

Barber, B. E., William, T., Grigg, M. J., Parameswaran, U., Piera, K. A., Price, R. N., Yeo, T. W. and Anstey, N. M. (2015) 'Parasite biomass-related inflammation, endothelial activation, microvascular dysfunction and disease severity in *vivax* malaria', *PLoS Pathogens*, 11(1), p. e1004558.

Barnwell, J. W., Asch, A. S., Nachman, R. L., Yamaya, M., Aikawa, M. and Ingravallo, P. (1989) 'A human 88-kD membrane glycoprotein (CD36) functions *in vitro* as a receptor for a cytoadherence ligand on *Plasmodium falciparum*-infected erythrocytes', *Journal of Clinical Investigation*, 84(3), pp. 765–772.

Barrera, V., Hiscott, P. S., Craig, A. G., White, V. A., Milner, D. A., Beare, N. A. V., MacCormick, I. J. C., Kamiza, S., Taylor, T. E., Molyneux, M. E. and Harding, S. P. (2015) 'Severity of retinopathy parallels the degree of parasite sequestration in the eyes and brains of Malawian children with fatal cerebral malaria', *Journal of Infectious Diseases*, 211(12), pp. 1977–1986.

Baruch, D. I., Ma, X. C., Singh, H. B., Bi, X., Pasloske, B. L. and Howard, R. J. (1997) 'Identification of a region of PfEMP1 that mediates adherence of *Plasmodium falciparum* infected erythrocytes to CD36: conserved function with variant sequence', *Blood*, 90(9), pp. 3766–3775.

Baruch, D. I., Pasloske, B. L., Singh, H. B., Bi, X., Ma, X. C., Feldman, M., Taraschi, T. F. and Howard, R. J. (1995) 'Cloning the *P. falciparum* gene encoding PfEMP1, a malarial variant antigen and adherence receptor on the surface of parasitized human erythrocytes', *Cell*, 82(1), pp. 77–87.

Beeson, J. and Brown, G. (2004) '*Plasmodium falciparum*–infected erythrocytes demonstrate dual specificity for adhesion to hyaluronic acid and

chondroitin sulfate A and have distinct adhesive', *Journal of Infectious Diseases*, 189, pp. 169–179.

Bengtsson, A., Joergensen, L., Rask, T. S., Olsen, R. W., Andersen, M. A., Turner, L., Theander, T. G., Hviid, L., Higgins, M. K., Craig, A., Brown, A. and Jensen, A. T. R. (2013) 'A novel domain cassette identifies *Plasmodium falciparum* PfEMP1 proteins binding ICAM-1 and is a target of cross-reactive, adhesion-inhibitory antibodies.', *Journal of Immunology*, 190(1), pp. 240–249.

Berendt, A. R., Simmons, D. L., Tansey, J., Newbold, C. I. and Marsh, K. (1989) 'Intercellular adhesion molecule-1 is an endothelial cell adhesion receptor for *Plasmodium falciparum*', *Nature*, 341(6237), pp. 57–59.

Berger, S. S., Turner, L., Wang, C. W., Petersen, J. E. V, Kraft, M., Lusingu, J. P. A., Mmbando, B., Marquard, A. M., Bengtsson, D. B. A. C., Hviid, L., Nielsen, M. A., Theander, T. G. and Lavstsen, T. (2013) '*Plasmodium falciparum* expressing Domain Cassette 5 Type PfEMP1 (DC5-PfEMP1) bind PECAM1', *PLoS ONE*, 8(7), p. e69117.

Bernabeu, M., Danziger, S. A., Avril, M., Vaz, M., Babar, P. H., Brazier, A. J., Herricks, T., Maki, J. N., Pereira, L., Mascarenhas, A., Gomes, E., Chery, L., Aitchison, J. D., Rathod, P. K. and Smith, J. D. (2016) 'Severe adult malaria is associated with specific PfEMP1 adhesion types and high parasite biomass', *Proceedings of the National Academy of Sciences*, 113(23), pp. E3270–E3279.

Bertin, G. I., Lavstsen, T., Guillonneau, F., Doritchamou, J., Wang, C. W., Jespersen, J. S., Ezimegnon, S., Fievet, N., Alao, M. J., Lalya, F., Massougbodji, A., Ndam, N. T., Theander, T. G. and Deloron, P. (2013) 'Expression of the Domain Cassette 8 *Plasmodium falciparum* erythrocyte membrane protein 1 is associated with Cerebral Malaria in Benin', *PLoS ONE*, 8(7), p. e68368.

Bertin, G. I., Sabbagh, A., Argy, N., Salnot, V., Ezinmegnon, S., Agbota, G., Ladipo, Y., Alao, J. M., Sagbo, G., Guillonneau, F. and Deloron, P. (2016) 'Proteomic analysis of *Plasmodium falciparum* parasites from patients with cerebral and uncomplicated malaria', *Scientific Reports*, 6, p. 26773.

Bhasin, V. K. and Trager, W. (1984) 'Gametocyte-forming and non-gametocyte-forming clones of *Plasmodium falciparum*', *American Journal of Tropical Medicine and Hygiene*, 33(4), pp. 534–537.

Bhatt, S., Weiss, D. J., Cameron, E., Bisanzio, D., Mappin, B., Dalrymple, U., Battle, K. E., Moyes, C. L., Henry, A., Eckhoff, P. A., Wenger, E. A., Briet, O., Penny, M. A., Smith, T. A., Bennett, A., Yukich, J., Eisele, T. P., Griffin, J. T., Fergus, C. A., Lynch, M., Lindgren, F., Cohen, J. M., Murray, C. L. J., Smith, D. L., Hay, S. I., Cibulskis, R. E. and Gething, P. W. (2015) 'The effect of malaria control on *Plasmodium falciparum* in Africa between 2000 and 2015', *Nature*, 526(7572), pp. 207–211.

Biggs, B.-A., Gooze, L., Wycherley, K., Wilkinson, D., Boyd, A. W., Forsyth, K. P., Edelman, L., Brown, G. and Leech, J. H. (1990) 'Knob- independent cytoadherence of *Plasmodium falciparum* to the leukocyte differentiation antigen CD36', *Journal of Experimental Medicine*, 171, pp. 1883–92.

Biswas, A. K., Hafiz, A., Banerjee, B., Kim, K. S., Datta, K. and Chitnis, C. E. (2007) '*Plasmodium falciparum* uses gC1qR/HABP1/p32 as a receptor to bind to vascular endothelium and for platelet-mediated clumping', *PLoS Pathogens*, 3(9), pp. 1271–1280.

Bouharoun-Tayoun, H., Attanath, P., Sabchareon, A., Chongsuphajaisiddhi, T. and Druilhe, P. (1990) 'Antibodies that protect humans against *Plasmodium falciparum* blood stages do not on their own inhibit parasite growth and invasion *in vitro*, but act in cooperation with monocytes.', *The Journal of Experimental Medicine*, 172(6), pp. 1633–41.

Bouharoun-Tayoun, H., Oeuvray, C., Lunel, F. and Druilhe, P. (1995) 'Mechanisms underlying the monocyte-mediated antibody-dependent killing of *Plasmodium falciparum* asexual blood stages.', *The Journal of Experimental Medicine*, 182(2), pp. 409–418.

Bouïs, D., Hospers, G. A. P., Meijer, C., Molema, G. and Mulder, N. H. (2001) 'Endothelium *in vitro*: A review of human vascular endothelial cell lines for blood vessel-related research', *Angiogenesis*, 4(2), pp. 91–102.

Bouwens, E. A. M., Stavenuiter, F. and Mosnier, L. O. (2013) 'Mechanisms of anticoagulant and cytoprotective actions of the protein C pathway', *Journal of Thrombosis and Haemostasis*, 11(Suppl.1), pp. 242–253.

Bowyer, P. W., Stewart, L. B., Aspeling-Jones, H., Mensah-Brown, H. E., Ahouidi, A. D., Amambua-Ngwa, A., Awandare, G. A. and Conway, D. J. (2015)

‘Variation in *Plasmodium falciparum* erythrocyte invasion phenotypes and merozoite ligand gene expression across different populations in areas of malaria endemicity’, *Infection and Immunity*, 83(6), pp. 2575–2582.

Brown, H., Turner, G., Rogerson, S., Tembo, M., Mwenechanya, J., Molyneux, M. and Taylor, T. (1999) ‘Cytokine expression in the brain in human cerebral malaria.’, *Journal of Infectious Diseases*, 180(5), pp. 1742–1746.

Bull, P. C. and Abdi, A. I. (2016) ‘The Role of PfEMP1 as targets of naturally acquired immunity to childhood malaria’, *Parasitology*, 143(2), pp. 171–186.

Bull, P. C., Berriman, M., Kyes, S., Quail, M. A., Hall, N., Kortok, M. M., Marsh, K. and Newbold, C. I. (2005) ‘*Plasmodium falciparum* variant surface antigen expression patterns during malaria’, *PLoS Pathogens*, 1(3), p. e26.

Bull, P. C., Lowe, B. S., Kortok, M., Molyneux, C. S., Newbold, C. I. and Marsh, K. (1998) ‘Parasite antigens on the infected red cell surface are targets for naturally acquired immunity to malaria.’, *Nature Medicine*, 4(3), pp. 358–60.

Cabrera, A., Neculai, D. and Kain, K. C. (2014) ‘CD36 and malaria: Friends or foes? A decade of data provides some answers’, *Trends in Parasitology*, 30(9), pp. 436–444.

Carvalho, P. A., Diez-Silva, M., Chen, H., Dao, M. and Suresh, S. (2013) ‘Cytoadherence of erythrocytes invaded by *Plasmodium falciparum*: Quantitative contact-probing of a human malaria receptor’, *Acta Biomaterialia*, 9(5), pp. 6349–6359.

Chan, J.-A., Fowkes, F. J. I. and Beeson, J. G. (2014) ‘Surface antigens of *Plasmodium falciparum*-infected erythrocytes as immune targets and malaria vaccine candidates.’, *Cellular and Molecular Life Sciences*, 71(19), pp. 3633–57.

Chan, J. A., Howell, K. B., Reiling, L., Ataide, R., Mackintosh, C. L., Fowkes, F. J. I., Petter, M., Chesson, J. M., Langer, C., Warimwe, G. M., Duffy, M. F., Rogerson, S. J., Bull, P. C., Cowman, A. F., Marsh, K. and Beeson, J. G. (2012) ‘Targets of antibodies against *Plasmodium falciparum*-infected erythrocytes in malaria immunity’, *Journal of Clinical Investigation*, 122(9), pp. 3227–3238.

Cheng, T., Liu, D., Griffin, J. H., Fernández, J. a, Castellino, F., Rosen, E. D., Fukudome, K. and Zlokovic, B. V (2003) ‘Activated protein C blocks p53-

mediated apoptosis in ischemic human brain endothelium and is neuroprotective.', *Nature medicine*, 9(3), pp. 338–342.

Chizzolini, C., Delaporte, E., Kaufmann, M. H., Akue, J. P., Verdini, A. S., Pessi, A. and Del Giudice, G. (1989) 'Age-related prevalence of antibody response against three different, defined *Plasmodium falciparum* antigens in children from the Haut-Ogooué province in Gabon', *Transactions of the Royal Society of Tropical Medicine and Hygiene*, 83(2), pp. 147–151.

Chotivanich, K., Udomsangpetch, R., Simpson, J. a, Newton, P., Pukrittayakamee, S., Looareesuwan, S. and White, N. J. (2000) 'Parasite multiplication potential and the severity of falciparum malaria.', *Journal of Infectious Diseases*, 181(3), pp. 1206–9.

Claessens, A., Adams, Y., Ghumra, A., Lindergard, G., Buchan, C. C., Andisi, C., Bull, P. C., Mok, S., Gupta, A. P., Wang, C. W., Turner, L., Arman, M., Raza, A., Bozdech, Z. and Rowe, J. A. (2012) 'A subset of group A-like *var* genes encodes the malaria parasite ligands for binding to human brain endothelial cells.', *Proceedings of the National Academy of Sciences of the United States of America*, 109(26), pp. E1772-81.

Claessens, A., Hamilton, W. L., Kekre, M., Otto, T. D., Faizullabhoy, A., Rayner, J. C. and Kwiatkowski, D. (2014) 'Generation of antigenic diversity in *Plasmodium falciparum* by structured rearrangement of *var* genes during mitosis', *PLoS Genetics*, 10(12).

Claessens, A. and Rowe, J. A. (2012) 'Selection of *Plasmodium falciparum* parasites for cytoadhesion to human brain endothelial cells.', *Journal of Visualized Experiments*, (59), p. e3122.

Cohen, S., McGregor, I. and Carrington, S. (1961) 'Gamma-Globulin and Aquired Immunity to Human Malaria', *Nature*, 192(4804), pp. 733–737.

Cojean, S., Jafari-Guemouri, S., Le Bras, J. and Durand, R. (2008) 'Cytoadherence characteristics to endothelial receptors ICAM-1 and CD36 of *Plasmodium falciparum* populations from severe and uncomplicated malaria cases.', *Parasite*, 15(2), pp. 163–169.

Combes, V., El-Assaad, F., Faille, D., Jambou, R., Hunt, N. H. and Grau, G. E. R. (2010) 'Microvesiculation and cell interactions at the brain-endothelial

interface in cerebral malaria pathogenesis', *Progress in Neurobiology*, 91(2), pp. 140–151.

Cowman, A. F., Berry, D. and Baum, J. (2012) 'The cellular and molecular basis for malaria parasite invasion of the human red blood cell', *Journal of Cell Biology*, 198(6), pp. 961–971.

Crabb, B. S., Cooke, B. M., Reeder, J. C., Waller, R. F., Caruana, S. R., Davern, K. M., Wickham, M. E., Brown, G. V., Coppel, R. L. and Cowman, A. F. (1997) 'Targeted gene disruption shows that knobs enable malaria-infected red cells to cytoadhere under physiological shear stress', *Cell*, 89(2), pp. 287–296.

Craig, A. G., Grau, G. E., Janse, C., Kazura, J. W., Milner, D., Barnwell, J. W., Turner, G. and Langhorne, J. (2012) 'The role of animal models for research on severe malaria', *PLoS Pathogens*, 8(2), p. e1002401.

Craig, A. G., Khairul, M. F. M. and Patil, P. R. (2012) 'Cytoadherence and severe malaria', *Malaysian Journal of Medical Sciences*, 19(2), pp. 5–18.

Crandall, I., Smith, H. and Sherman, I. W. W. (1991) '*Plasmodium falciparum*: The effect of pH and Ca2+ concentration on the *in vitro* cytoadherence of infected erythrocytes to amelanotic melanoma cells', *Experimental Parasitology*, 73(3), pp. 362–368.

Crompton, P. D., Moebius, J., Waisberg, M., Garver, L. S., Miller, L. H., Barillas, C. and Pierce, S. K. (2014) 'Malaria immunity in man and mosquito: insights into unsolved mysteries of a deadly infectious disease', *Annual Review of Immunology*, 32, pp. 157–187.

Cserti-Gazdewich, C. M., Dhabangi, A., Musoke, C., Ssewanyana, I., Ddungu, H., Nakiboneka-Ssenabulya, D., Nabukeera-Barungi, N., Mpimbaza, A. and Dzik, W. H. (2012) 'Cytoadherence in paediatric malaria: ABO blood group, CD36, and ICAM1 expression and severe *Plasmodium falciparum* infection', *British Journal of Haematology*, 159(2), pp. 223–236.

Cuhadar, S., Koseoglu, M., Atay, A. and Dirican, A. (2013) 'The effect of storage time and freeze-thaw cycles on the stability of serum samples', *Biochemia Medica*, 23(1), pp. 70–77.

Dahlbäck, M., Lavstsen, T., Salanti, A., Hviid, L., Arnot, D. E., Theander, T. G. and Nielsen, M. a (2007) 'Changes in *var* gene mRNA levels during

erythrocytic development in two phenotypically distinct *Plasmodium falciparum* parasites.', *Malaria Journal*, 6(78).

Day, N. P., Hien, T. T., Schollaardt, T., Loc, P. P., Chuong, L. V, Chau, T. T., Mai, N. T., Phu, N. H., Sinh, D. X., White, N. J. and Ho, M. (1999) 'The prognostic and pathophysiologic role of pro- and antiinflammatory cytokines in severe malaria', *Journal of Infectious Diseases*, 180(4), pp. 1288–1297.

Deane, R., LaRue, B., Sagare, A. P., Castellino, F. J., Zhong, Z. and Zlokovic, B. V (2009) 'Endothelial protein C receptor-assisted transport of activated protein C across the mouse blood-brain barrier.', *Journal of Cerebral Blood Flow and Metabolism*, 29(1), pp. 25–33.

Deans, A. M., Lyke, K. E., Thera, M. A., Plowe, C. V., Kone, A., Doumbo, O. K., Kai, O., Marsh, K., Mackinnon, M. J., Raza, A. and Rowe, J. A. (2006) 'Low multiplication rates of African *Plasmodium falciparum* isolates and lack of association of multiplication rate and red blood cell selectivity with malaria virulence', *American Journal of Tropical Medicine and Hygiene*, 74(4), pp. 554–563.

Deans, A. M., Nery, S., Conway, D. J., Kai, O., Marsh, K. and Rowe, J. A. (2007) 'Invasion pathways and malaria severity in Kenyan *Plasmodium falciparum* clinical isolates', *Infection and Immunity*, 75(6), pp. 3014–3020.

Delley, V., Bouvier, P., Breslow, N., Doumbo, O., Sagara, I., Diakite, M., Mauris, A., Dolo, A. and Rougemont, A. (2000) 'What does a single determination of malaria parasite density mean? A longitudinal survey in Mali', *Tropical Medicine and International Health*, 5(6), pp. 404–412.

Deroost, K., Pham, T. T., Opdenakker, G. and Van den Steen, P. E. (2016) 'The immunological balance between host and parasite in malaria', *FEMS Microbiology Reviews*, 40(2), pp. 208–257.

Desowitz, R. S., Elm, J. and Alpers, M. P. (1993) '*Plasmodium falciparum*-specific immunoglobulin G (IgG), IgM, and IgE antibodies in paired maternal-cord sera from East Sepik province, Papua New Guinea', *Infection and Immunity*, 61(3), pp. 988–993.

Dobbs, K. R. and Dent, A. E. (2016) '*Plasmodium* malaria and antimalarial antibodies in the first year of life', *Parasitology*, 143, pp. 129–138.

Dondorp, A. M., Ince, C., Charunwatthana, P., Hanson, J., van Kuijen, A., Faiz, M. A., Rahman, M. R., Hasan, M., Bin Yunus, E., Ghose, A., Ruangveerayut, R., Limmathurotsakul, D., Mathura, K., White, N. J. and Day, N. P. (2008) 'Direct *in vivo* assessment of microcirculatory dysfunction in severe falciparum malaria', *Journal of Infectious Diseases*, 197(1), pp. 79–84.

Dondorp, A. M., Pongponratn, E. and White, N. J. (2004) 'Reduced microcirculatory flow in severe falciparum malaria: Pathophysiology and electron-microscopic pathology', *Acta Tropica*, 89(3), pp. 309–317.

Dorovini-Zis, K., Prameya, R. and Bowman, P. D. (1991) 'Culture and characterisation of microvascular endothelial cells derived from human brain', *Laboratory Investigation*, 64(3), pp. 425–436.

Duah, N. O., Miles, D. J. C., Whittle, H. C. and Conway, D. J. (2010) 'Acquisition of antibody isotypes against *Plasmodium falciparum* blood stage antigens in a birth cohort', *Parasite Immunology*, 32(2), pp. 125–134.

Dzikowski, R., Frank, M. and Deitsch, K. (2006) 'Mutually exclusive expression of virulence genes by malaria parasites is regulated independently of antigen production', *PLoS Pathogens*, 2(3), pp. 0184–0194.

English, M., Sauerwein, R., Waruiru, C., Mosobo, M., Obiero, J., Lowe, B. and Marsh, K. (1997) 'Acidosis in severe childhood malaria.', *QJM*, 90(4), pp. 263–270.

English, M., Waruiru, C., Amukoye, E., Murphy, S., Crawley, J., Mwangi, I., Peshu, N. and Marsh, K. (1996) 'Deep breathing in children with severe malaria: Indicator of metabolic acidosis and poor outcome', *American Journal of Tropical Medicine and Hygiene*, 55(5), pp. 521–524.

Esamai, F., Mining, S., Forsberg, P. and Lewis, D. H. D. H. (2001) 'A comparison of brain, core and skin temperature in children with complicated and uncomplicated malaria', *Journal of Tropical Pediatrics*, 47(3), pp. 170–175.

Falk, N., Kaestli, M., Qi, W., Ott, M., Baea, K., Cortés, A. and Beck, H.-P. (2009) 'Analysis of *Plasmodium falciparum var* genes expressed in children from Papua New Guinea.', *Journal of infectious diseases*, 200, pp. 347–356.

Farnert, A., Snounou, G., Rooth, I. and Bjorkman, A. (1997) 'Daily Dynamics of *Plasmodium falciparum* Subpopulations in Asymptomatic Children

in a Holoendemic Area', *American Journal of Tropical Medicine and Hygiene*, 56(5), pp. 538–547.

Fastman, Y., Noble, R., Recker, M. and Dzikowski, R. (2012) 'Erasing the epigenetic memory and beginning to switch-the onset of antigenic switching of *var* genes in *Plasmodium falciparum*', *PLoS ONE*, 7(3), p. e34168.

Franke-Fayard, B., Fonager, J., Braks, A., Khan, S. M. and Janse, C. J. (2010) 'Sequestration and tissue accumulation of human malaria parasites: Can we learn anything from rodent models of malaria?', *PLoS Pathogens*, 6(9), p. e1001032.

Frankland, S., Elliott, S. R., Yosaatmadja, F., Beeson, J. G., Rogerson, S. J., Adisa, A. and Tilley, L. (2007) 'Serum lipoproteins promote efficient presentation of the malaria virulence protein PfEMP1 at the erythrocyte surface', *Eukaryotic Cell*, 6(9), pp. 1584–1594.

Fried, M. and Duffy, P. E. (2015) 'Designing a VAR2CSA-based vaccine to prevent placental malaria', *Vaccine*, 33(52), pp. 7483–7488.

Fry, A. E., Ghansa, A., Small, K. S., Palma, A., Auburn, S., Diakite, M., Green, A., Campino, S., Teo, Y. Y., Clark, T. G., Jeffreys, A. E., Wilson, J., Jallow, M., Sisay-Joof, F., Pinder, M., Griffiths, M. J., Peshu, N., Williams, T. N., Newton, C. R., Marsh, K., Molyneux, M. E., Taylor, T. E., Koram, K. A., Oduro, A. R., Rogers, W. O., Rockett, K. A., Sabeti, P. C. and Kwiatkowski, D. P. (2009) 'Positive selection of a CD36 nonsense variant in sub-Saharan Africa, but no association with severe malaria phenotypes', *Human Molecular Genetics*, 18(14), pp. 2683–2692.

Ganguly, A. K., Ranjan, P., Kumar, A. and Bhavesh, N. S. (2015) 'Dynamic association of PfEMP1 and KAHRP in knobs mediates cytoadherence during *Plasmodium* invasion.', *Scientific Reports*, 5, p. 8617.

Gardner, M. J., Hall, N., Fung, E., White, O., Berriman, M., Hyman, R. W., Carlton, J. M., Pain, A., Nelson, K. E., Bowman, S., Paulsen, I. T., James, K., Eisen, J. A., Rutherford, K., Salzberg, S. L., Craig, A., Kyes, S., Chan, M.-S., Nene, V., Shallom, S. J., Suh, B., Peterson, J., Angiuoli, S., Pertea, M., Allen, J., Selengut, J., Haft, D., Mather, M. W., Vaidya, A. B., Martin, D. M. A., Fairlamb, A. H., Fraunholz, M. J., Roos, D. S., Ralph, S. A., McFadden, G. I., Cummings, L. M., Subramanian, G. M., Mungall, C., Venter, J. C., Carucci, D. J., Hoffman, S. L., Newbold, C., Davis, R. W.,

Fraser, C. M. and Barrell, B. (2002) ‘Genome sequence of the human malaria parasite *Plasmodium falciparum*.’, *Nature*, 419(6906), pp. 498–511.

Garraud, O., Mahanty, S. and Perraut, R. (2003) ‘Malaria-specific antibody subclasses in immune individuals: A key source of information for vaccine design’, *Trends in Immunology*, 24(1), pp. 30–35.

Gerald, N., Mahajan, B. and Kumar, S. (2011) ‘Mitosis in the human malaria parasite *Plasmodium falciparum*’, *Eukaryotic Cell*, 10(4), pp. 474–482.

Ghebrehiwet, B., Cebadamora, C., Tantral, L., Jesty, J. and Peerschke, E. I. B. (2006) ‘gC1qR/p33 serves as a molecular bridge between the complement and contact activation systems and is an important catalyst in inflammation’, *Advances in Experimental Medicine and Biology*, 586, pp. 95–105.

Ghebrehiwet, B., Lim, B. L., Peerschke, E. I. B., Willis, A. C. and Reid, K. B. M. (1994) ‘Isolation, cDNA cloning, and overexpression of a 33-kD cell surface glycoprotein that binds to the globular “heads” of C1q.’, *Journal of Experimental Medicine*, 179(6), pp. 1809–1821.

Gillrie, M. R., Avril, M., Brazier, A. J., Davis, S. P., Stins, M. F., Smith, J. D. and Ho, M. (2015) ‘Diverse functional outcomes of *Plasmodium falciparum* ligation of EPCR: Potential implications for malarial pathogenesis’, *Cellular Microbiology*, 17(12), pp. 1883–1899.

Goel, S., Palmkvist, M., Moll, K., Joannin, N., Lara, P., R Akhouri, R., Moradi, N., Öjemalm, K., Westman, M., Angeletti, D., Kjellin, H., Lehtiö, J., Blixt, O., Ideström, L., Gahmberg, C. G., Storry, J. R., Hult, A. K., Olsson, M. L., von Heijne, G., Nilsson, I. and Wahlgren, M. (2015) ‘RIFINs are adhesins implicated in severe *Plasmodium falciparum* malaria’, *Nature Medicine*, 21(4), pp. 314–317.

Gomez-Escobar, N., Amambua-Ngwa, A., Walther, M., Okebe, J., Ebonyi, A. and Conway, D. J. (2010) ‘Erythrocyte invasion and merozoite ligand gene expression in severe and mild *Plasmodium falciparum* malaria.’, *Journal of Infectious Diseases*, 201(3), pp. 444–52.

Gonçalves, B. P., Huang, C.-Y., Morrison, R., Holte, S., Kabyemela, E., Prevots, D. R., Fried, M. and Duffy, P. E. (2014) ‘Parasite burden and severity of malaria in Tanzanian children’, *New England Journal of Medicine*, 370, pp. 1799–1808.

Gonçalves, R. M., Scopel, K. K. G., Bastos, M. S. and Ferreira, M. U. (2012) 'Cytokine balance in human malaria: Does *Plasmodium vivax* elicit more inflammatory responses than *Plasmodium falciparum*?', *PLoS ONE*, 7(9), p. e44394.

Gray, C., McCormick, C., Turner, G. and Craig, A. (2003) 'ICAM-1 can play a major role in mediating *P. falciparum* adhesion to endothelium under flow', *Molecular and Biochemical Parasitology*, 128(2), pp. 187–193.

Groux, H., Perraut, R., Garraud, O., Poingt, J. P. and Gysin, J. (1990) 'Functional characterization of the antibody-mediated protection against blood stages of *Plasmodium falciparum* in the monkey Saimiri sciureus', *European Journal of Immunology*, 20(10), pp. 2317–2323.

Gullingsrud, J., Saveria, T., Amos, E., Duffy, P. E. and Oleinikov, A. V. (2013) 'Structure-function-immunogenicity studies of PfEMP1 domain DBL2bPF11_0521, a Malaria parasite ligand for ICAM-1', *PLoS ONE*, 8(4), p. e61323.

Haldar, K. and Mohandas, N. (2009) 'Malaria, erythrocytic infection, and anemia', *Hematology. American Society of Hematology Education Program*, pp. 87–93.

Hanson, J., Lam, S. W. K., Mahanta, K. C., Pattnaik, R., Alam, S., Mohanty, S., Hasan, M. U., Hossain, A., Charunwatthana, P., Chotivanich, K., Maude, R. J., Kingston, H., Day, N. P., Mishra, S., White, N. J. and Dondorp, A. M. (2012) 'Relative contributions of macrovascular and microvascular dysfunction to disease severity in falciparum malaria', *Journal of Infectious Diseases*, 206(4), pp. 571–579.

Hansson, H. H., Turner, L., Møller, L., Wang, C. W., Minja, D. T. R., Gesase, S., Mmbando, B., Bygbjerg, I. C., Theander, T. G., Lusingu, J. P. A., Alifrangis, M. and Lavstsen, T. (2015) 'Haplotypes of the endothelial protein C receptor (EPCR) gene are not associated with severe malaria in Tanzania.', *Malaria Journal*, 14(474).

Hartgers, F. C. and Yazdanbakhsh, M. (2006) 'Co-infection of helminths and malaria: Modulation of the immune responses to malaria', *Parasite Immunology*, 28(10), pp. 497–506.

Hasday, J. D., Bannerman, D., Sakarya, S., Cross, A. S., Singh, I. S., Howard, D., Drysdale, B. E. and Goldblum, S. E. (2001) 'Exposure to febrile temperature modifies endothelial cell response to tumor necrosis factor-alpha.', *Journal of Applied Physiology*, 90(1), pp. 90–98.

Heddini, A., Chen, Q., Obiero, J., Kai, O., Fernandez, V., Marsh, K., Muller, W. A. and Wahlgren, M. (2001) 'Binding of *Plasmodium falciparum*-infected erythrocytes to soluble platelet endothelial cell adhesion molecule-1 (PECAM-1/CD31): Frequent recognition by clinical isolates', *American Journal of Tropical Medicine and Hygiene*, 65(1), pp. 47–51.

Heddini, A., Pettersson, F., Kai, O., Shafi, J., Obiero, J., Chen, Q., Barragan, A., Wahlgren, M. and Marsh, K. (2001) 'Fresh Isolates from Children with Severe *Plasmodium falciparum* Malaria Bind to Multiple Receptors', *Society*, 69(9), pp. 5849–5856.

Ho, M. and Singh, B. (1991) 'Clinical correlates of *in vitro Plasmodium falciparum* cytoadherence', *Infection and Immunity*, 59(3), pp. 873–878.

Hochman, S. and Kim, K. (2009) 'The impact of HIV and malaria coinfection: What is known and suggested venues for further study.', *Interdisciplinary Perspectives on Infectious Diseases*, 2009, p. 617954.

Hochman, S., Madaline, T., Seydel, K., Taylor, T., Milner, D., S., L. and K., K. (2013) 'Fatal pediatric cerebral malaria is associated with intravascular inflammation and coagulation that is exacerbated by HIV-1 co-infection', *American Journal of Tropical Medicine and Hygiene*, 89(5), p. 457.

Holt, P. G. and Jones, C. A. (2000) 'The development of the immune system during pregnancy and early life.', *Allergy*, 55(8), pp. 688–697.

Horrocks, P., Pinches, R., Christodoulou, Z., Kyes, S. A. and Newbold, C. I. (2004) 'Variable *var* transition rates underlie antigenic variation in malaria.', *Proceedings of the National Academy of Sciences of the United States of America*, 101(30), pp. 11129–11134.

Houde, D., Peng, Y., Berkowitz, S. A. and Engen, J. R. (2010) 'Post-translational modifications differentially affect IgG1 conformation and receptor binding', *Molecular and Cellular Proteomics*, 9(8), pp. 1716–1728.

Hughes, K. R., Biagini, G. A. and Craig, A. G. (2010) 'Continued

cytoadherence of *Plasmodium falciparum* infected red blood cells after antimalarial treatment', *Molecular and Biochemical Parasitology*, 169(2), pp. 71–78.

Hunt, N. H. and Grau, G. E. (2003) 'Cytokines: Accelerators and brakes in the pathogenesis of cerebral malaria', *Trends in Immunology*, 24(9), pp. 491–499.

Hviid, L. (2005) 'Naturally acquired immunity to *Plasmodium falciparum* malaria in Africa', *Acta Tropica*, 95(3), pp. 270–275.

Hviid, L. (2009) 'Unraveling the Impact of Malaria Exposure Before Birth', *PLoS Medicine*, 6(7), p. e1000117.

Hviid, L. (2010) 'The role of *Plasmodium falciparum* variant surface antigens in protective immunity and vaccine development', *Human vaccines*, 6(1), pp. 84–89.

Hviid, L., Barfod, L. and Fowkes, F. J. I. (2015) 'Trying to remember: Immunological B cell memory to malaria', *Trends in Parasitology*, 31(3), pp. 89–94.

Hviid, L. and Jensen, A. T. R. (2015) 'PfEMP1 - a parasite protein family of key importance in *Plasmodium falciparum* malaria immunity and pathogenesis', *Advances in Parasitology*, 88, pp. 51–84.

Idro, R., Kakooza-Mwesige, A., Balyejjussa, S., Mirembe, G., Mugasha, C., Tugumisirize, J. and Byarugaba, J. (2010) 'Severe neurological sequelae and behaviour problems after cerebral malaria in Ugandan children.', *BMC research notes*, 3, p. 104.

Jain, V., Singh, P. P., Silawat, N., Patel, R., Saxena, A., Bharti, P. K., Shukla, M., Biswas, S. and Singh, N. (2010) 'A preliminary study on pro- and anti-inflammatory cytokine profiles in *Plasmodium vivax* malaria patients from central zone of India', *Acta Tropica*, 113(3), pp. 263–268.

Jallow, M., Casals-Pascual, C., Ackerman, H., Walther, B., Walther, M., Pinder, M., Sisay-Joof, F., Usen, S., Jallow, M., Abubakar, I., Olaosebikan, R., Jobarteh, A., Conway, D. J., Bojang, K. and Kwiatkowski, D. (2012) 'Clinical features of severe malaria associated with death: A 13-year observational study in The Gambia', *PLoS ONE*, 7(9), p. e45645.

Janes, J. H., Wang, C. P., Levin-Edens, E., Vigan-Womas, I., Guillotte, M., Melcher, M., Mercereau-Puijalon, O. and Smith, J. D. (2011) ‘Investigating the host binding signature on the *Plasmodium falciparum* PfEMP1 protein family’, *PLoS Pathogens*, 7(5), p. e1002032.

Jaspan, H. B., Lawn, S. D., Safrit, J. T. and Bekker, L.-G. (2006) ‘The maturing immune system: implications for development and testing HIV-1 vaccines for children and adolescents.’, *AIDS*, 20(4), pp. 483–494.

Jensen, A. T. R., Magistrado, P., Sharp, S., Joergensen, L., Lavstsen, T., Chiucchiuini, A., Salanti, A., Vestergaard, L. S., Lusingu, J. P., Hermsen, R., Sauerwein, R., Christensen, J., Nielsen, M. a, Hviid, L., Sutherland, C., Staalsoe, T. and Theander, T. G. (2004) ‘*Plasmodium falciparum* associated with severe childhood malaria preferentially expresses PfEMP1 encoded by group A *var* genes.’, *Journal of Experimental Medicine*, 199(9), pp. 1179–1190.

Jensen, J. B. (1978) ‘Concentration from continous culture of erythrocytes infected with trophozoites and schizonts of *Plasmodium falciparum*’, *American Journal of Tropical Medicine and Hygiene*, 27(6), pp. 1274–1276.

Jespersen, J. S., Wang, C. W., Mkumbaye, S. I., Daniel, T. R., Theander, T. G. and Lavstsen, T. (2016) ‘*Plasmodium falciparum var* genes expressed in severe malaria characteristically encode CIDRα1 domains’, *EMBO Molecular Medicine*, p. e201606188.

Joannin, N., Abhiman, S., Sonnhammer, E. L. and Wahlgren, M. (2008) ‘Sub-grouping and sub-functionalization of the RIFIN multi-copy protein family.’, *BMC genomics*, 9, p. 19.

Joergensen, L., Bengtsson, D. C., Bengtsson, A., Ronander, E., Berger, S. S., Turner, L., Dalgaard, M. B., Cham, G. K. K., Victor, M. E., Lavstsen, T., Theander, T. G., Arnot, D. E. and Jensen, A. T. R. (2010) ‘Surface co-expression of two different PfEMP1 antigens on single *Plasmodium falciparum*-infected erythrocytes facilitates binding to ICAM1 and PECAM1’, *PLoS Pathogens*, 6(9).

John, C. C., Kutamba, E., Mugarura, K. and Opoka, R. O. (2010) ‘Adjunctive therapy for cerebral malaria and other severe forms of *Plasmodium falciparum* malaria’, *Expert Reviews in Anti-Infection Therapy*, 8(9), pp. 997–1008.

Kaestli, M., Cockburn, I. a, Cortés, A., Baea, K., Rowe, J. A. and Beck, H.-P. (2006) 'Virulence of malaria is associated with differential expression of *Plasmodium falciparum var* gene subgroups in a case-control study.', *Journal of infectious diseases*, 193(11), pp. 1567–1574.

Kalmbach, Y., Rottmann, M., Kombila, M., Kremsner, P. G., Beck, H.-P. and Kun, J. F. J. (2010) 'Differential *var* gene expression in children with malaria and antidromic effects on host gene expression.', *Journal of Infectious Diseases*, 202(2), pp. 313–317.

Kassim, O. O., Ako-Anai, K. A., Torimiro, S. E., Hollowell, G. P., Okoye, V. C. and Martin, S. K. (2000) 'Inhibitory factors in breastmilk, maternal and infant sera against *in vitro* growth of *Plasmodium falciparum* malaria parasite', *Journal of Tropical Pediatrics*, 46(2), pp. 92–96.

Kern, P., Hemmer, C. J., Van Damme, J., Gruss, H. J. and Dietrich, M. (1989) 'Elevated tumor necrosis factor alpha and interleukin-6 serum levels as markers for complicated *Plasmodium falciparum* malaria', *The American journal of medicine*, 87, pp. 139–143.

Kilejian, A., Sharma, Y., Karoui, H. and Naslund, L. (1986) 'Histidine-rich domain of the knob protein of the human malaria parasite *Plasmodium falciparum*', *Proceedings of the National Academy of Sciences of the United States of America*, 83, pp. 7938–7941.

Kirchgatter, K. and Portillo, H. D. a (2002) 'Association of severe noncerebral *Plasmodium falciparum* malaria in Brazil with expressed PfEMP1 DBL1 alpha sequences lacking cysteine residues.', *Molecular medicine*, 8(1), pp. 16–23.

Kraemer, S. M. and Smith, J. D. (2003) 'Evidence for the importance of genetic structuring to the structural and functional specialization of the *Plasmodium falciparum var* gene family', *Molecular Microbiology*, 50(5), pp. 1527–1538.

Kraemer, S. M. and Smith, J. D. (2006) 'A family affair: *var* genes, PfEMP1 binding, and malaria disease', *Current Opinion in Microbiology*, 9(4), pp. 374–380.

Krishna, S. (2012) 'Adjunctive management of malaria', *Current Opinion*

in Infectious Diseases, 25(5), pp. 484–488.

Krych-Goldberg, M., Moulds, J. M. and Atkinson, J. P. (2002) 'Human complement receptor type 1 (CR1) binds to a major malarial adhesin', *Trends in Molecular Medicine*, 8(11), pp. 531–537.

Kwiatkowski, D. (1989) 'Febrile temperatures can synchronize the growth of *Plasmodium falciparum in vitro*', 169, pp. 357–361.

Kwiatkowski, D., Hill, A. V. S., Sambou, I., Twumasi, P., Castracane, J. and Manogue, K. R. (1990) 'TNF concentration in fatal cerebral, non-fatal cerebral, and uncomplicated *Plasmodium falciparum* malaria', *Lancet*, 336, pp. 1201–1204.

Kyes, S. A., Kraemer, S. M. and Smith, J. D. (2007) 'Antigenic variation in *Plasmodium falciparum*: Gene organization and regulation of the *var* multigene family', *Eukaryotic Cell*, 6(9), pp. 1511–1520.

Kyes, S. A., Rowe, J. A., Kriek, N. and Newbold, C. I. (1999) 'Rifins: a second family of clonally variant proteins expressed on the surface of red cells infected with *Plasmodium falciparum*.', *Proceedings of the National Academy of Sciences of the United States of America*, 96(16), pp. 9333–9338.

Kyes, S., Pinches, R. and Newbold, C. (2000) 'A simple RNA analysis method shows *var* and *rif* multigene family expression patterns in *Plasmodium falciparum*', *Molecular and Biochemical Parasitology*, 105(2), pp. 311–315.

Kyriacou, H. M., Stone, G. N., Challis, R. J., Raza, A., Lyke, K. E., Thera, M. A., Kone, A. K., Doumbo, O. K., Plowe, C. V. and Rowe, J. A. (2006) 'Differential *var* gene transcription in *Plasmodium falciparum* isolates from patients with cerebral malaria compared to hyperparasitaemia', *Molecular and Biochemical Parasitology*, 150(2), pp. 211–218.

Langreth, S. G. and Peterson, E. (1985) 'Pathogenicity, stability, and immunogenicity of a knobless clone of *Plasmodium falciparum* in Colombian owl monkeys', *Infection and Immunity*, 47(3), pp. 760–766.

Laszik, Z., Mitro, A., Taylor Jr., F. B., Ferrell, G. and Esmon, C. T. (1997) 'Human protein C receptor is present primarily on endothelium of large blood vessels: implications for the control of the protein C pathway', *Circulation*, 96(10), pp. 3633–3640.

Lau, C. K. Y., Turner, L., Jespersen, J. S., Lowe, E. D., Petersen, B., Wang, C. W., Petersen, J. E. V, Lusingu, J., Theander, T. G., Lavstsen, T. and Higgins, M. K. (2015) 'Structural conservation despite huge sequence diversity allows EPCR binding by the PfEMP1 family implicated in severe childhood malaria', *Cell Host and Microbe*, 17(1), pp. 118–129.

Lavazec, C., Sanyal, S. and Templeton, T. J. (2007) 'Expression switching in the stevor and Pfmc-2TM superfamilies in *Plasmodium falciparum*', *Molecular Microbiology*, 64(6), pp. 1621–1634.

Lavstsen, T., Salanti, A., Jensen, A. T. R., Arnot, D. E. and Theander, T. G. (2003) 'Sub-grouping of *Plasmodium falciparum* 3D7 *var* genes based on sequence analysis of coding and non-coding regions.', *Malaria Journal*, 2(27).

Lavstsen, T., Turner, L., Saguti, F., Magistrado, P., Rask, T. S., Jespersen, J. S., Wang, C. W., Berger, S. S., Baraka, V., Marquard, A. M., Seguin-Orlando, A., Willerslev, E., Gilbert, M. T. P., Lusingu, J. and Theander, T. G. (2012) '*Plasmodium falciparum* erythrocyte membrane protein 1 domain cassettes 8 and 13 are associated with severe malaria in children', *Proceedings of the National Academy of Sciences*, 109(26), pp. E1791–E1800.

Lee, D. H., Kim, J. W., Jeon, S. Y., Park, B. K. and Han, B. G. (2010) 'Proteomic analysis of the effect of storage temperature on human serum', *Annals of Clinical and Laboratory Science*, 40(1), pp. 61–70.

van Leeuwen, H. C. and O'Hare, P. (2001) 'Retargeting of the mitochondrial protein p32/gC1Qr to a cytoplasmic compartment and the cell surface.', *Journal of Cell Science*, 114, pp. 2115–2123.

Liu, W., Li, Y., Shaw, K. S., Learn, G. H., Plenderleith, L. J., Malenke, J. A., Sundararaman, S. A., Ramirez, M. A., Crystal, P. A., Smith, A. G., Bibollet-Ruche, F., Ayouba, A., Locatelli, S., Esteban, A., Mouacha, F., Guichet, E., Butel, C., Ahuka-Mundeke, S., Inogwabini, B.-I., Ndjango, J.-B. N., Speede, S., Sanz, C. M., Morgan, D. B., Gonder, M. K., Kranzusch, P. J., Walsh, P. D., Georgiev, A. V, Muller, M. N., Piel, A. K., Stewart, F. A., Wilson, M. L., Pusey, A. E., Cui, L., Wang, Z., Färnert, A., Sutherland, C. J., Nolder, D., Hart, J. A., Hart, T. B., Bertolani, P., Gillis, A., LeBreton, M., Tafon, B., Kiyang, J., Djoko, C. F., Schneider, B. S., Wolfe, N. D., Mpoudi-Ngole, E., Delaporte, E., Carter, R., Culleton, R. L., Shaw, G. M., Rayner, J.

C., Peeters, M., Hahn, B. H. and Sharp, P. M. (2014) 'African origin of the malaria parasite *Plasmodium vivax*.', *Nature Communications*, 5, p. 3346.

Lyke, K. E., Burges, R., Cissoko, Y., Sangare, L., Dao, M., Diarra, I., Kone, A., Harley, R., Plowe, C., Doumbo, O. and Sztein, M. (2004) 'Serum levels of the proinflammatory IL-8 , IL-10 , tumor necrosis factor alpha , and IL-12 (p70) in Malian children with severe *Plasmodium falciparum* malaria and matched uncomplicated malaria or healthy controls', *Infection and Immunity*, 72(10), pp. 5630–5637.

MacKintosh, C. L., Beeson, J. G. and Marsh, K. (2004) 'Clinical features and pathogenesis of severe malaria', *Trends in Parasitology*, 20(12), pp. 597–603.

MacPherson, G. G., Warrell, M. J., White, N. J., Looareesuwan, S. and Warrell, D. A. (1985) 'Human cerebral malaria. A quantitative ultrastructural analysis of parasitized erythrocyte sequestration.', *American Journal of Pathology*, 119(3), pp. 385–401.

Madkhali, A. M., Alkurbi, M. O., Szestak, T., Bengtsson, A., Patil, P. R., Wu, Y., Alharthi, S., Jensen, A. T. R., Pleass, R. and Craig, A. G. (2014) 'An analysis of the binding characteristics of a panel of recently selected ICAM-1 binding *Plasmodium falciparum* patient isolates', *PLoS ONE*, 9(10), p. e111518.

Maitland, K. and Marsh, K. (2004) 'Pathophysiology of severe malaria in children', *Acta Tropica*, 90, pp. 131–140.

Malhotra, I., Dent, A., Mungai, P., Wamachi, A., Ouma, J. H., Narum, D. L., Muchiri, E., Tisch, D. J. and King, C. L. (2009) 'Can prenatal malaria exposure produce an immune tolerant phenotype?: A prospective birth cohort study in Kenya', *PLoS Medicine*, 6(7), p. e1000116.

Manning, L., Laman, M., Law, I., Bona, C., Aipit, S., Teine, D., Warrell, J., Rosanas-Urgell, A., Lin, E., Kiniboro, B., Vince, J., Hwaiwhanje, I., Karunajeewa, H., Michon, P., Siba, P., Mueller, I. and Davis, T. M. E. (2011) 'Features and prognosis of severe malaria caused by *Plasmodium falciparum*, *Plasmodium vivax* and mixed *Plasmodium* species in Papua New Guinean children', *PLoS ONE*, 6(12), p. e29203.

Marinkovic, M., Diez-Silva, M., Pantic, I., Fredberg, J. J., Suresh, S. and Butler, J. P. (2009) 'Febrile temperature leads to significant stiffening of

Plasmodium falciparum parasitized erythrocytes.', *American Journal of Physiology. Cell Physiology*, 296(1), pp. C59–C64.

Marsh, K., Forster, D., Waruiru, C., Mwangi, I., Winstanley, M., Marsh, V., Newton, C., Winstanley, P., Warn, P. and Peshu, N. (1995) 'Indicators of life-threatening malaria in African children.', *New England Journal of Medicine*, 332(21), pp. 1399–1404.

Marsh, K., Marsh, V. M., Brown, J., Whittle, H. C. and Greenwood, B. M. (1988) '*Plasmodium falciparum*: The behavior of clinical isolates in an *in vitro* model of infected red blood cell sequestration', *Experimental Parasitology*, 65(2), pp. 202–208.

Marsh, K., Otoo, L., Hayes, R. J., Carson, D. C. and Greenwood, B. M. (1989) 'Antibodies to blood stage antigens of *Plasmodium falciparum* in rural gambians and their relation to protection against infection', *Transactions of the Royal Society of Tropical Medicine and Hygiene*, 83(3), pp. 293–303.

Maurus, C. F., Schmidt, D., Schneider, M. K. J., Turina, M. I., Seebach, J. D. and Zünd, G. (2003) 'Hypoxia and reoxygenation do not upregulate adhesion molecules and natural killer cell adhesion on human endothelial cells *in vitro*.', *European Journal of Cardio-thoracic Surgery*, 23(6), pp. 976–983.

Mayor, A., Hafiz, A., Bassat, Q., Rovira-Vallbona, E., Sanz, S., Machevo, S., Aguilar, R., Cistero, P., Sigauque, B., Menendez, C., Alonso, P. L. and Chitnis, C. E. (2011) 'Association of severe malaria outcomes with platelet-mediated clumping and adhesion to a novel host receptor', *PLoS ONE*, 6(4), p. e19422.

Medina, P., Navarro, S., Bonet, E., Martos, L., Estellés, A., Bertina, R. M., Vos, H. L. and España, F. (2014) 'Functional analysis of two haplotypes of the human endothelial protein C receptor gene', *Arteriosclerosis, Thrombosis, and Vascular Biology*, 34(3), pp. 684–690.

Mensah-Brown, H. E., Amoako, N., Abugri, J., Stewart, L. B., Agongo, G., Dickson, E. K., Ofori, M. F., Stoute, J. A., Conway, D. J. and Awandare, G. A. (2015) 'Analysis of erythrocyte invasion mechanisms of *Plasmodium falciparum* clinical isolates across 3 malaria-endemic areas in Ghana', *Journal of Infectious Diseases*, 212(8), pp. 1288–1297.

Menschikowski, M., Hagelgans, A., Eisenhofer, G. and Siegert, G. (2009)

‘Regulation of endothelial protein C receptor shedding by cytokines is mediated through differential activation of MAP kinase signaling pathways’, *Experimental Cell Research*, 315(15), pp. 2673–2682.

Mibei, E. K., Otieno, W. O., Orago, A. S. S. and Stoute, J. A. (2008) ‘Distinct pattern of class and subclass antibodies in immune complexes of children with cerebral malaria and severe malarial anaemia’, *Parasite Immunology*, 30(6–7), pp. 334–341.

Miller, L. H., Ackerman, H. C., Su, X. and Wellems, T. E. (2013) ‘Malaria biology and disease pathogenesis: insights for new treatments.’, *Nature Medicine*, 19(2), pp. 156–167.

Miller, L. H., Baruch, D. I., Marsh, K. and Doumbo, O. K. (2002) ‘The pathogenic basis of malaria’, *Nature*, 415, pp. 673–679.

Milner, D. A., Montgomery, J., Seydel, K. B. and Rogerson, S. J. (2008) ‘Severe malaria in children and pregnancy: an update and perspective’, *Trends in Parasitology*, 24(12), pp. 590–595.

Milner, D. A., Whitten, R. O., Kamiza, S., Carr, R., Liomba, G., Dzamalala, C., Seydel, K. B., Molyneux, M. E. and Taylor, T. E. (2014) ‘The systemic pathology of cerebral malaria in African children.’, *Frontiers in Cellular and Infection Microbiology*, 4, p. 104.

Mirghani, H. A., Eltahir, H. G., A-elgadir, T. M., Mirghani, Y. A., Elbashir, M. I. and Adam, I. (2011) ‘Cytokine profiles in children with severe *Plasmodium falciparum* malaria in an area of unstable malaria transmission in central sudan’, *Journal of Tropical Pediatrics*, 57(5), pp. 392–395.

Molineaux, L., Trauble, M., Collins, W. E., Jeffery, G. M. and Dietz, K. (2002) ‘Malaria therapy reinoculation data suggest individual variation immune response and independent acquisition of antiparasitic immunities of an innate and antitoxic immunities’, *Transactions of the Royal Society of Tropical Medicine and Hygiene*, 96, pp. 205–209.

Molyneux, M. E., Engelmann, H., Taylor, T. E., Wirima, J. J., Aderka, D., Wallach, D. and Grau, G. E. (1993) ‘Circulating plasma receptors for tumour necrosis factor in Malawian children with severe falciparum malaria.’, *Cytokine*, 5(6), pp. 604–609.

Mosnier, L. O. and Lavstsen, T. (2016) 'The role of EPCR in the pathogenesis of severe malaria', *Thrombosis Research*, 141, pp. S46–S49.

Moussiliou, A., Alao, M. J., Denoeud-Ndam, L., Tahar, R., Ezimegnon, S., Sagbo, G., Amoussou, A., Luty, A. J. F., Deloron, P. and Tuikue Ndam, N. (2014) 'High plasma levels of soluble Endothelial Protein C Receptor are associated with increased mortality among children With cerebral malaria in Benin.', *Journal of Infectious Diseases*, 211, pp. 1484–1488.

Moxon, C. A., Chisala, N. V., Wassmer, S. C., Taylor, T. E., Seydel, K. B., Molyneux, M. E., Faragher, B., Kennedy, N., Toh, C. H., Craig, A. G. and Heyderman, R. S. (2014) 'Persistent endothelial activation and inflammation after *Plasmodium falciparum* infection in Malawian children', *Journal of Infectious Diseases*, 209(4), pp. 610–615.

Moxon, C. A., Wassmer, S. C., Milner, D. A., Chisala, N. V., Taylor, T. E., Seydel, K. B., Molyneux, M. E., Faragher, B., Esmon, C. T., Downey, C., Toh, C. H., Craig, A. G. and Heyderman, R. S. (2013) 'Loss of endothelial protein C receptors links coagulation and inflammation to parasite sequestration in cerebral malaria in African children', *Blood*, 122(5), pp. 842–851.

Mugasa, J., Qi, W., Rusch, S., Rottman, M. and Beck, H.-P. (2012) 'Genetic diversity of expressed *Plasmodium falciparum var* genes from Tanzanian children with severe malaria', *Malaria Journal*, 11(230).

Mutabingwa, T. K., Bolla, M. C., Li, J. L., Domingo, G. J., Li, X., Fried, M. and Duffy, P. E. (2005) 'Maternal malaria and gravidity interact to modify infant susceptibility to malaria', *PLoS Medicine*, 2(12), pp. 1260–1268.

Naka, I., Patarapotikul, J., Hananantachai, H., Imai, H. and Ohashi, J. (2014) 'Association of the endothelial protein C receptor (PROCR) rs867186-G allele with protection from severe malaria.', *Malaria Journal*, 13(105).

Ndam, N. G. T., Salanti, A., Bertin, G., Dahlbäck, M., Fievet, N., Turner, L., Gaye, A., Theander, T. and Deloron, P. (2005) 'High level of *var2csa* transcription by *Plasmodium falciparum* isolated from the placenta.', *Journal of Infectious Diseases*, 192(2), pp. 331–335.

Newbold, C., Warn, P., Black, G., Berendt, A., Craig, A., Snow, B. O. B., Msobo, M. and Peshu, N. (1997) 'Receptor-specific adhesion and clinical disease

in *Plasmodium falciparum*', *The American Journal of Tropical Medicine and Hygiene*, 57(4), pp. 389–398.

Niang, M., Bei, A. K., Madnani, K. G., Pelly, S., Dankwa, S., Kanjee, U., Gunalan, K., Amaladoss, A., Yeo, K. P., Bob, N. S., Malleret, B., Duraisingh, M. T. and Preiser, P. R. (2014) 'STEVOR is a *Plasmodium falciparum* erythrocyte binding protein that mediates merozoite invasion and rosetting', *Cell Host and Microbe*, 16(1), pp. 81–93.

Niang, M., Xue, Y. Y. and Preiser, P. R. (2009) 'The *Plasmodium falciparum* STEVOR multigene family mediates antigenic variation of the infected erythrocyte', *PLoS Pathogens*, 5(2), p. e1000307.

Nogueira, P. A., Wunderlich, G., Shugiro Tada, M., D'Arc Neves Costa, J., Menezes, M. J., Scherf, A. and Pereira-da-Silva, L. H. (2002) '*Plasmodium falciparum*: Analysis of transcribed *var* gene sequences in natural isolates from the Brazilian Amazon region', *Experimental Parasitology*, 101(2–3), pp. 111–120.

Nunes-Silva, S., Dechavanne, S., Moussiliou, A., Pstrąg, N., Semblat, J.-P., Gangnard, S., Tuikue-Ndam, N., Deloron, P., Chêne, A. and Gamain, B. (2015) 'Beninese children with cerebral malaria do not develop humoral immunity against the IT4-VAR19-DC8 PfEMP1 variant linked to EPCR and brain endothelial binding', *Malaria Journal*, 14(1), p. 493.

O'Carroll, S. J., Kho, D. T., Wiltshire, R., Nelson, V., Rotimi, O., Johnson, R., Angel, C. E. and Graham, E. S. (2015) 'Pro-inflammatory TNFalpha and IL-1beta differentially regulate the inflammatory phenotype of brain microvascular endothelial cells', *Journal of Neuroinflammation*, 12, p. 131.

Oakley, M. S. M., Kumar, S., Anantharaman, V., Zheng, H., Mahajan, B., Haynes, J. D., Moch, J. K., Fairhurst, R., McCutchan, T. F. and Aravind, L. (2007) 'Molecular factors and biochemical pathways induced by febrile temperature in intraerythrocytic *Plasmodium falciparum* parasites', *Infection and Immunity*, 75(4), pp. 2012–2025.

Oberli, A., Zurbrügg, L., Rusch, S., Brand, F., Butler, M. E., Day, J. L., Cutts, E. E., Lavstsen, T., Vakonakis, I. and Beck, H.-P. (2016) '*Plasmodium falciparum* PHIST proteins contribute to cytoadherence and anchor PfEMP1 to the host cell

cytoskeleton', *Cellular Microbiology*, 18(10), pp. 1415–1428.

Ochola, L. B., Siddondo, B. R., Ocholla, H., Nkya, S., Kimani, E. N., Williams, T. N., Makale, J. O., Liljander, A., Urban, B. C., Bull, P. C., Szestak, T., Marsh, K. and Craig, A. G. (2011) 'Specific receptor usage in *Plasmodium falciparum* cytoadherence is associated with disease outcome', *PLoS ONE*, 6(3), pp. 1–9.

Ockenhouse, C. F., Ho, M., Tandon, N. N., Vanseventer, G. A., Shaw, S., White, N. J., Jamieson, G. A., Chulay, J. D. and Webster, H. K. (1991) 'Molecular basis of sequestration in severe and uncomplicated *Plasmodium falciparum* malaria: - Differential adhesion of infected erythrocytes to CD36 and ICAM-1', *Journal of Infectious Diseases*, 164(1), pp. 163–169.

Ockenhouse, C. F., Tandon, N. N., Magowan, C., Jamieson, G. A. and Chulay, J. D. (1989) 'Identification of a platelet membrane glycoprotein as a falciparum malaria sequestration receptor.', *Science*, 243(4897), pp. 1469–71.

Ofori, M. F., Dodoo, D., Staalsoe, T., Kurtzhals, J. A. L., Koram, K., Theander, T. G., Akanmori, B. D. and Hviid, L. (2002) 'Malaria-induced acquisition of antibodies to *Plasmodium falciparum* variant surface antigens', *Infection and Immunity*, 70(6), pp. 2982–2988.

Oleinikov, A. V., Amos, E., Frye, I. T., Rossnagle, E., Mutabingwa, T. K., Fried, M. and Duffy, P. E. (2009) 'High throughput functional assays of the variant antigen PfEMP1 reveal a single domain in the 3D7 *Plasmodium falciparum* genome that binds ICAM1 with high affinity and is targeted by naturally acquired neutralizing antibodies', *PLoS Pathogens*, 5(4), p. e1000386.

Opi, D. H., Ochola, L. B., Tendwa, M., Siddondo, B. R., Ocholla, H., Fanjo, H., Ghumra, A., Ferguson, D. J. P., Rowe, J. A. and Williams, T. N. (2014) 'Mechanistic studies of the negative epistatic malaria-protective interaction between sickle cell trait and a+thalassemia', *EBioMedicine*, 1(1), pp. 29–36.

Phiri, H., Montgomery, J., Molyneux, M. and Craig, A. (2009) 'Competitive endothelial adhesion between *Plasmodium falciparum* isolates under physiological flow conditions.', *Malaria Journal*, 8(214).

Phyo, A. P., Nkhoma, S., Stepniewska, K., Ashley, E. A., Nair, S., McGready, R., Moo, C. L., Al-Saai, S., Dondorp, A. M., Lwin, K. M., Singhasivanon, P., Day, N. P. J., White, N. J., Anderson, T. J. C. and Nosten, F. (2012) 'Emergence of

artemisinin-resistant malaria on the western border of Thailand: A longitudinal study', *Lancet*, 379(9830), pp. 1960–1966.

Pintao, M. C., Roshani, S., De Visser, M. C. H., Tieken, C., Tanck, M. W. T., Wichers, I. M., Meijers, J. C. M., Rosendaal, F. R., Middeldorp, S. and Reitsma, P. H. (2011) 'High levels of protein C are determined by PROCR haplotype 3', *Journal of Thrombosis and Haemostasis*, 9(5), pp. 969–976.

Ponsford, M. J., Medana, I. M., Prapansilp, P., Hien, T. T., Lee, S. J., Dondorp, A. M., Esiri, M. M., Day, N. P. J., White, N. J. and Turner, G. D. H. (2012) 'Sequestration and microvascular congestion are associated with coma in human cerebral malaria', *Journal of Infectious Diseases*, 205(4), pp. 663–671.

Portugal, S., Pierce, S. K. and Crompton, P. D. (2013) 'Young lives lost as B Cells falter: What we are learning about antibody responses in malaria', *Journal of Immunology*, 190, pp. 3039–3046.

Pouvelle, B., Fusaï, T., Lépolard, C., Gysin, J., Fusai, T. and Lepolard, C. (1998) 'Biological and biochemical characteristics of cytoadhesion of *Plasmodium falciparum*-infected erythrocytes to chondroitin-4-sulfate.', *Infection and Immunity*, 66(10), pp. 4950–4956.

Prudêncio, M., Rodriguez, A. and Mota, M. M. (2006) 'The silent path to thousands of merozoites: the *Plasmodium* liver stage.', *Nature Reviews. Microbiology*, 4(11), pp. 849–856.

Rahimi, B. A., Thakkinstian, A., White, N. J., Sirivichayakul, C., Dondorp, A. M. and Chokejindachai, W. (2014) 'Severe vivax malaria: a systematic review and meta-analysis of clinical studies since 1900.', *Malaria Journal*, 13(481).

Rao, L. V. M., Esmon, C. T. and Pendurthi, U. R. (2014) 'Endothelial cell protein C receptor : a multiliganded and multifunctional receptor', *Blood*, 124(10), pp. 1553–1563.

Rask, T. S., Hansen, D. A., Theander, T. G., Pedersen, A. G. and Lavstsen, T. (2010) '*Plasmodium falciparum* erythrocyte membrane protein 1 diversity in seven genomes - divide and conquer', *PLoS Computational Biology*, 6(9), p. e1000933.

Rénia, L., Wu Howland, S., Claser, C., Charlotte Gruner, A., Suwanarusk, R., Hui Teo, T., Russell, B. and Ng, L. (2012) 'Cerebral malaria: Mysteries at the

blood-brain barrier', *Virulence*, 3(2), pp. 183–191.

Riewald, M., Petrovan, R. J., Donner, A., Mueller, B. M. and Ruf, W. (2002) 'Activation of endothelial cell protease activated receptor 1 by the protein C pathway', *Science*, 296(5574), pp. 1880–1882.

Robinson, B. A., Welch, T. L. and Smith, J. D. (2003) 'Widespread functional specialization of *Plasmodium falciparum* erythrocyte membrane protein 1 family members to bind CD36 analysed across a parasite genome', *Molecular Microbiology*, 47(5), pp. 1265–1278.

Robson, K. J. H., Walliker, D., Creasey, A., McBride, J., Beale, G. and Wilson, R. J. M. (1992) 'Cross-contamination of *Plasmodium* cultures', *Parasitology Today*, 8(2), pp. 38–39.

Rogerson, S., Chaiyaroj, S., Ng, K., Reeder, J. and Brown, G. (1995) 'Chondroitin sulfate A is a cell surface receptor for *Plasmodium falciparum*-infected erythrocytes', *Journal of Experimental Medicine*, 182, pp. 15–20.

Rogerson, S. J., Tembenu, R., Doban, C. and Molyneux, M. E. (1999) 'Cytoadherence characteristics of *Plasmodium falciparum*-infected erythrocytes from Malawian children with severe and uncomplicated malaria', *American Journal of Tropical Medicine and Hygiene*, 61(3), pp. 467–472.

Rottmann, M., Lavstsen, T., Mugasa, J. P., Kaestli, M., Jensen, A. T. R., Muller, D., Theander, T. and Beck, H. P. (2006) 'Differential expression of *var* gene groups is associated with morbidity caused by *Plasmodium falciparum* infection in Tanzanian children', *Infection and Immunity*, 74(7), pp. 3904–3911.

Rowe, J. A., Claessens, A., Corrigan, R. A. and Arman, M. (2009) 'Adhesion of *Plasmodium falciparum*-infected erythrocytes to human cells: molecular mechanisms and therapeutic implications', *Expert Reviews in Molecular Medicine*, 11, p. e16.

Rowe, J. A., Handel, I. G., Thera, M. A., Deans, A.-M., Lyke, K. E., Kone, A., Diallo, D. A., Raza, A., Kai, O., Marsh, K., Plowe, C. V, Doumbo, O. K. and Moulds, J. M. (2007) 'Blood group O protects against severe *Plasmodium falciparum* malaria through the mechanism of reduced rosetting', *Proceedings of the National Academy of Sciences*, 104(44), pp. 17471–17476.

Rowe, J. A., Moulds, J. M., Newbold, C. I. and Miller, L. H. (1997) '*P.*

falciparum rosetting mediated by a parasite-variant erythrocyte membrane protein and complement-receptor 1.', *Nature*, 388(6639), pp. 292–295.

Ruwende, C., Khoo, S. C., Snow, R. W., Yates, S. N. R., Kwiatkowski, D., Gupta, S., Warn, P., Allsopp, C. E. M., Gilbert, S. C., Peschu, N., Newbold, C. I., Greenwood, B. M., Marsh, K. and Hill, A. V. S. (1995) 'Natural selection of hemi- and heterozygotes for G6PD deficiency in Africa by resistance to severe malaria', *Nature*, 376(6537), pp. 246–249.

Salanti, A., Dahlback, M., Turner, L., Nielsen, M. a, Barfod, L., Magistrado, P., Jensen, A. T. R., Lavstsen, T., Ofori, M. F., Marsh, K., Hviid, L., Theander, T. G., Dahlbäck, M., Turner, L., Nielsen, M. a, Barfod, L., Magistrado, P., Jensen, A. T. R., Lavstsen, T., Ofori, M. F., Marsh, K., Hviid, L. and Theander, T. G. (no date) 'Evidence for the involvement of VAR2CSA in pregnancy-associated malaria', *Journal of Experimental Medicine*, 200(9), pp. 1197–1203.

Sampath, S., Brazier, A. J., Avril, M., Bernabeu, M., Vigdorovich, V., Mascarenhas, A., Gomes, E., Sather, D. N., Esmon, C. T. and Smith, J. D. (2015) '*Plasmodium falciparum* adhesion domains linked to severe malaria differ in blockade of endothelial protein C receptor', *Cellular Microbiology*, 17(12), pp. 1868–1882.

Scherer, E. F., Cantarini, D. G., Siqueira, R., Ribeiro, E. B., Braga, T. M., Honório-França, A. C. and França, E. L. (2016) 'Cytokine modulation of human blood viscosity from vivax malaria patients', *Acta Tropica*, 158, pp. 139–147.

Schuldt, K., Ehmen, C., Evans, J., May, J., Ansong, D., Sievertsen, J., Muntau, B., Ruge, G., Agbenyega, T. and Horstmann, R. D. (2014) 'Endothelial protein C receptor gene variants not associated with severe malaria in Ghanaian children', *PLoS ONE*, 9(12), p. e115770.

Sehgal, V. M., Siddiqui, W. A. and Alpers, M. (1989) 'A seroepidemiological study to evaluate the role of passive maternal immunity to malaria in infants', *Transactions of the Royal Society of Tropical Medicine and Hygiene*, 83, pp. 105–106.

Seydel, K. B., Milner, D. A, Kamiza, S. B., Molyneux, M. E. and Taylor, T. E. (2006) 'The distribution and intensity of parasite sequestration in comatose Malawian children.', *Journal of Infectious Diseases*, 194(2), pp. 208–215.

Shabani, E., Opoka, R. O., Bangirana, P., Park, G. S., Vercellotti, G. M., Guan, W., Hodges, J. S., Lavstsen, T. and John, C. C. (2016) 'The endothelial protein C receptor rs867186-GG genotype is associated with increased soluble EPCR and could mediate protection against severe malaria', *Scientific Reports*, 6, p. 27084.

Shah, A., Unger, E., Bain, M. D., Bruce, R., Bodkin, J., Ginnetti, J., Wang, W. C., Seon, B., Stewart, C. C. and Evans, S. S. (2002) 'Cytokine and adhesion molecule expression in primary human endothelial cells stimulated with fever-range hyperthermia', *International Journal of Hyperthermia*, 18(6), pp. 534–551.

Shanks, G. D. and White, N. J. (2013) 'The activation of vivax malaria hypnozoites by infectious diseases', *Lancet Infectious Diseases*, 13, pp. 900–906.

Sherman, I. W., Eda, S. and Winograd, E. (2003) 'Cytoadherence and sequestration in *Plasmodium falciparum*: Defining the ties that bind', *Microbes and Infection*, 5(10), pp. 897–909.

Sherman, I. W. and Valdez, E. (1989) '*In vitro* cytoadherence of *Plasmodium falciparum*-infected erythrocytes to melanoma cells: factors affecting adhesion', *Parasitology*, 98, pp. 359–369.

Silvestrini, F., Alano, P. and Williams, J. L. (2000) 'Commitment to the production of male and female gametocytes in the human malaria parasite *Plasmodium falciparum*', *Parasitology*, 121, pp. 465–471.

Singh, B. and Daneshvar, C. (2013) 'Human infections and detection of *Plasmodium knowlesi*', *Clinical Microbiology Reviews*, 26(2), pp. 165–184.

Smith, J. D., Chitnis, C. E., Craig, A. G., Roberts, D. J., Hudson-Taylor, D. E., Peterson, D. S., Pinches, R., Newbold, C. I. and Miller, L. H. (1995) 'Switches in expression of *Plasmodium falciparum var* genes correlate with changes in antigenic and cytoadherent phenotypes of infected erythrocytes', *Cell*, 82(1), pp. 101–110.

Smith, J. D., Craig, A. G., Kriek, N., Hudson-Taylor, D., Kyes, S., Fagan, T., Fagen, T., Pinches, R., Baruch, D. I., Newbold, C. I. and Miller, L. H. (2000b) 'Identification of a *Plasmodium falciparum* intercellular adhesion molecule-1 binding domain: a parasite adhesion trait implicated in cerebral malaria.', *Proceedings of the National Academy of Sciences of the United States of America*, 97(4), pp. 1766–1771.

Smith, J. D., Rowe, J. A., Higgins, M. K. and Lavstsen, T. (2013) 'Malaria's deadly grip: Cytoadhesion of *Plasmodium falciparum*-infected erythrocytes', *Cellular Microbiology*, 15(12), pp. 1976–1983.

Smith, J. D., Subramanian, G., Gamain, B., Baruch, D. I. and Miller, L. H. (2000a) 'Classification of adhesive domains in the *Plasmodium falciparum* Erythrocyte Membrane Protein 1 family', *Molecular and Biochemical Parasitology*, 110(2), pp. 293–310.

Snow, R. W. and Marsh, K. (1998) 'New insights into the epidemiology of malaria relevant for disease control.', *British Medical Bulletin*, 54(2), pp. 293–309.

Subramani, R., Quadt, K., Jeppesen, A. E., Hempel, C., Vang, E. and Hassenkam, T. (2015) '*Plasmodium falciparum*-infected erythrocyte knob density is linked to the PfEMP1 variant expressed', *mBio*, 6(5), pp. e01456-15.

Targett, G. A. T. (1970) 'Antibody response to *Plasmodium falciparum*: Comparisons of immunoglobulin concentrations, antibody titres and the antigenicity of different asexual forms of the parasite', *Clinical and Experimental Immunology*, 7, pp. 501–517.

Taylor, D. W., Parra, M., Chapman, G. B., Stearns, M. E., Rener, J., Aikawa, M., Uni, S., Aley, S. B., Panton, L. J. and Howard, R. J. (1987) 'Localization of *Plasmodium falciparum* histidine-rich protein 1 in the erythrocyte skeleton under knobs', *Molecular and Biochemical Parasitology*, 25(2), pp. 165–174.

Taylor, T. E., Fu, W. J., Carr, R. A., Whitten, R. O., Mueller, J. G., Fosiko, N. G., Lewallen, S., Liomba, N. G. and Molyneux, M. E. (2004) 'Differentiating the pathologies of cerebral malaria by postmortem parasite counts.', *Nature Medicine*, 10(2), pp. 143–145.

Taylor, T. E. and Molyneux, M. E. (2015) 'The pathogenesis of pediatric cerebral malaria: Eye exams, autopsies, and neuroimaging', *Annals of the New York Academy of Sciences*, 1342(1), pp. 44–52.

Tebo, A. E., Kremsner, P. G. and Luty, A. J. (2001) '*Plasmodium falciparum*: a major role for IgG3 in antibody-dependent monocyte-mediated cellular inhibition of parasite growth *in vitro*.', *Experimental Parasitology*, 98(1), pp. 20–28.

Tembo, D. L., Nyoni, B., Murikoli, R. V., Mukaka, M., Milner, D. A., Berriman, M., Rogerson, S. J., Taylor, T. E., Molyneux, M. E., Mandala, W. L., Craig, A. G. and Montgomery, J. (2014) 'Differential PfEMP1 expression is associated with cerebral malaria pathology', *PLoS Pathogens*, 10(12), p. e1004537.

Tilly, A.-K., Thiede, J., Metwally, N., Lubiana, P., Bachmann, A., Roeder, T., Rockliffe, N., Lorenzen, S., Tannich, E., Gutsmann, T. and Bruchhaus, I. (2015) 'Type of *in vitro* cultivation influences cytoadhesion, knob structure, protein localization and transcriptome profile of *Plasmodium falciparum*', *Scientific Reports*, 5, p. 16766.

Trager, W. (1993) 'Resistance and cross-contamination of culture lines of *Plasmodium falciparum*', *Parasitology Today*, 9(4), p. 127.

Travassos, M. A., Coulibaly, D., Bailey, J. A., Niangaly, A., Adams, M., Nyunt, M. M., Ouattara, A., Lyke, K. E., Laurens, M. B., Pablo, J., Jasinskas, A., Nakajima, R., Berry, A. A., Takala-Harrison, S., Kone, A. K., Kouriba, B., Rowe, J. A., Doumbo, O. K., Thera, M. A., Laufer, M. K., Felgner, P. L. and Plowe, C. V (2015) 'Differential recognition of terminal extracellular *Plasmodium falciparum* VAR2CSA domains by sera from multigravid, malaria-exposed Malian women.', *American Journal of Tropical Medicine and Hygiene*, 92(6), pp. 1190–1194.

Treutiger, C. J., Heddini, A., Fernandez, V., Muller, W. A. & and Wahlgren, M. (1997) 'PECAM-1/CD31, an endothelial receptor for binding *Plasmodium falciparum*-infected erythrocytes', *Nature Medicine*, 3(12), pp. 1405–1408.

Turner, G. D. H., Morrison, H., Jones, M., Davis, Timothy M. E. Looareesuwan, S., Buley, I. D., Gatter, K. C., Newbold, C., Pukritayakamee, S., Nagachinta, B., White, N. J. and Berendt, A. R. (1994) 'Immunohistochemical study of the pathology of fatal malaria', *American Journal of Pathology*, 145(5), pp. 1057–1069.

Turner, L., Lavstsen, T., Berger, S. S., Wang, C. W., Petersen, J. E. V, Avril, M., Brazier, A. J., Freeth, J., Jespersen, J. S., Nielsen, M. a, Magistrado, P., Lusingu, J., Smith, J. D., Higgins, M. K. and Theander, T. G. (2013) 'Severe malaria is associated with parasite binding to endothelial protein C receptor.', *Nature*, 498(7455), pp. 502–505.

Turner, L., Lavstsen, T., Mmbando, B. P., Wang, C. W., Magistrado, P. A.,

Vestergaard, L. S., Ishengoma, D. S., Minja, D. T. R., Lusingu, J. P. and Theander, T. G. (2015) 'IgG antibodies to Endothelial Protein C receptor-binding cysteine-rich interdomain region domains of *Plasmodium falciparum* erythrocyte membrane protein 1 are acquired early in life in individuals exposed to malaria', *Infection and Immunity*, 83(8), pp. 3096–3103.

Udeinya, I. J., Graves, P. M., Carter, R., Aikawa, M. and Miller, L. H. (1983) '*Plasmodium falciparum*: effect of time in continuous culture on binding to human endothelial cells and amelanotic melanoma cells.', *Experimental Parasitology*, 56, pp. 207–214.

Udeinya, I. J., Schmidt, J. A., Aikawa, M., Miller, L. H. and Green, I. (1981) 'Falciparum malaria infected erythrocytes specifically bind to cultured human endothelial cells.', *Science*, 213(4507), pp. 555–557.

Udomsangpetch, R., Pipitaporn, B., Silamut, K., Pinches, R., Kyes, S., Looareesuwan, S., Newbold, C. and White, N. J. (2002) 'Febrile temperatures induce cytoadherence of ring-stage *Plasmodium falciparum*-infected erythrocytes.', *Proceedings of the National Academy of Sciences of the United States of America*, 99(18), pp. 11825–11829.

Viebig, N. K., Wulbrand, U., Förster, R., Andrews, K. T., Lanzer, M., Knolle, P. A. and Fo, R. (2005) 'Direct activation of human endothelial cells by *Plasmodium falciparum*-infected erythrocytes', *Infection and Immunity*, 73(6), pp. 3271–3277.

Walther, M., Woodruff, J., Edele, F., Jeffries, D., Tongren, J. E., King, E., Andrews, L., Bejon, P., Gilbert, S. C., De Souza, J. B., Sinden, R., Hill, A. V. S. and Riley, E. M. (2006) 'Innate immune responses to human malaria: heterogeneous cytokine responses to blood-stage *Plasmodium falciparum* correlate with parasitological and clinical outcomes.', *Journal of Immunology*, 177(8), pp. 5736–5745.

Wambua, S., Mwangi, T. W., Kortok, M., Uyoga, S. M., Macharia, A. W., Mwacharo, J. K., Weatherall, D. J., Snow, R. W., Marsh, K. and Williams, T. N. (2006) 'The effect of α+-thalassaemia on the incidence of malaria and other diseases in children living on the coast of Kenya', *PLoS Medicine*, 3(5), pp. 643–651.

Wang, C. W. and Hviid, L. (2015) ‘Rifins, rosetting, and red blood cells’, *Trends in Parasitology*, 31(7), pp. 285–286.

Warimwe, G. M., Fegan, G., Musyoki, J. N., Newton, C. R. J. C., Opiyo, M., Githinji, G., Andisi, C., Menza, F., Kitsao, B., Marsh, K. and Bull, P. C. (2012) ‘Prognostic indicators of life-threatening malaria are associated with distinct parasite variant antigen profiles.’, *Science Translational Medicine*, 4(129), p. 129ra45.

Warimwe, G. M., Keane, T. M., Fegan, G., Musyoki, J. N., Newton, C. R. J. C., Pain, A., Berriman, M., Marsh, K. and Bull, P. C. (2009) ‘*Plasmodium falciparum var* gene expression is modified by host immunity.’, *Proceedings of the National Academy of Sciences of the United States of America*, 106(51), pp. 21801–21806.

Wassmer, S. C., Combes, V., Candal, F. J., Juhan-vague, I. and Grau, G. E. (2006) ‘Platelets potentiate brain endothelial alterations induced by *Plasmodium falciparum*’, *Infection and Immunity*, 74(1), pp. 645–653.

Wassmer, S. C., Moxon, C. A., Taylor, T., Grau, G. E., Molyneux, M. E. and Craig, A. G. (2011) ‘Vascular endothelial cells cultured from patients with cerebral or uncomplicated malaria exhibit differential reactivity to TNF’, *Cellular Microbiology*, 13(2), pp. 198–209.

Weksler, B., Romero, I. A. and Couraud, P.-O. (2013) ‘The hCMEC/D3 cell line as a model of the human blood brain barrier.’, *Fluids and barriers of the CNS*, 10(1), p. 16.

White, N. J., Looareesuwan, S., Phillips, R. E., Warrell, D. A., Chanthavanich, P. and Pongpaew, P. (1985) ‘Pathophysiological and Prognostic Significance of Cerebrospinal-Fluid Lactate in Cerebral Malaria’, *The Lancet*, 325(8432), pp. 776–778.

White, N. J., Turner, G. D. H., Medana, I. M., Dondorp, A. M. and Day, N. P. J. (2010) ‘The murine cerebral malaria phenomenon’, *Trends in Parasitology*, 26(1), pp. 11–15.

WHO (2014) ‘Severe malaria’, *Tropical Medicine and International Health*, 19(Suppl. I), pp. 7–131.

WHO (2015) ‘World Malaria Report’.

Xiao, L., Yang, C., Dorovini-Zis, K., Tandon, N. N., Ades, E. W., Lal, A. A. and

Udhayakumar, V. (1996) ‘*Plasmodium falciparum*: Involvement of additional receptors in the cytoadherence of infected erythrocytes to microvascular endothelial cells’, *Experimental Parasitology*, 84, pp. 42–55.

Yone, C. L. R. P., Kremsner, P. G. and Luty, A. J. F. (2005) ‘Immunoglobulin G isotype responses to erythrocyte surface-expressed variant antigens of *Plasmodium falciparum* predict protection from malaria in African children’, *Infection and Immunity*, 73(4), pp. 2281–2287.

Zeyrek, F. Y., Kurcer, M. A., Zeyrek, D. and Simsek, Z. (2006) ‘Parasite density and serum cytokine levels in *Plasmodium vivax* malaria in Turkey’, *Parasite Immunology*, 28(5), pp. 201–207.

Ziegelstein, R. C., He, C. and Hu, Q. (2004) ‘Hypoxia/reoxygenation stimulates Ca2+-dependent ICAM-1 mRNA expression in human aortic endothelial cells.’, *Biochemical and Biophysical Research Communications*, 322(1), pp. 68–73.