THE UNIVERSITY *of* EDINBURGH

Investigating the role of C1QBP for *Plasmodium falciparum* infected erythrocyte binding to human brain microvascular endothelial cells

Nouhoum DIALLO

A thesis submitted in partial fulfilment of the degree of Master of Philosophy

University of Edinburgh

2025

I. Abstract

Cerebral malaria (CM) is characterised by the adhesion and sequestration of *Plasmodium falciparum* infected erythrocytes (IEs) in the human brain microvascular endothelial cells (HBECs). IEs can adhere to immortalised human brain endothelial cells (hCMEC/D3) *in vitro*. This provides a reliable model for the investigation of interactions between IEs and human brain endothelium. Sequestration is mediated by interactions between members of the *P. falciparum* erythrocyte membrane protein 1(PfEMP1) family and receptors on the endothelial membrane. CM is associated with expression of specific PfEMP1 subtypes containing domain cassettes (DCs) 8 and 13. However, the major endothelial receptor for parasites exhibiting these ligands remains controversial but may include endothelial protein C receptor (EPCR) and intercellular adhesion molecule 1 (ICAM1). Previous work suggests complement C1q binding protein (C1QBP) as a potential binding partner for IEs, but this has rarely been studied. In this thesis, I used a) HBECs and immunofluorescence assays to determine the cellular localisation of C1QBP and b) adhesion assays to determine the role of C1QBP in IE (IT4VAR19, HB3VAR03, 9197VAR27, and PfKE08) adhesion to hCMEC/D3.

Firstly, I validated the endothelial nature of hCMEC/D3 with specific endothelial cell markers such as von Willebrand factor (vWF), PECAM1/CD31 (Platelet endothelial cell adhesion molecule 1), Dil-Ac-LDL (Dil-Acetylated- low density lipoprotein), and SMA (Smooth muscle actin). As expected hCMEC/D3 exhibited positive staining for vWF, PECAM1/CD31, Dil-Ac-LDL, and a negative staining for SMA. Therefore, I can use hCMEC/D3 as an endothelial cell to determine the cellular localisation of C1QBP. Resting and TNFα-activated hCMEC/D3 showed intracellular staining for C1QBP, but cell surface staining was not observed. However, after incubation with soluble C1QBP or normal human plasma (which contains soluble C1QBP), hCMEC/D3 did exhibit positive surface membrane expression of C1QBP. In addition, I observed that primary HBECs constitutively express C1QBP but not immortalised HBECs. These results show that primary HBECs may exhibit different surface receptors compared to immortalised HBECs.

Secondly, I investigated whether C1QBP can promote IE binding to hCMEC/D3. In static binding assays to purified receptors, IT4VAR19-IE showed significant binding to C1QBP, TSP (thrombospondin) and EPCR (endothelial protein C receptor). In adhesion inhibition assays, I found that a monoclonal antibody (mAb) to human EPCR blocked the interaction between

IT4VAR19-IE and hCMEC/D3, whereas a mAb and polyclonal (pAb) antibody to human C1QBP did not. HB3VAR03-IE did not interact with C1QBP and other potential endothelial cell receptors, while 9197VAR27 and PFKE08-IE did not also bind to C1QBP but significantly adhered to EPCR. These data do not support a role for C1QBP as a major endothelial cell cytoadhesion receptor for the four IEs lines studied here.

In conclusion, C1QBP is not constitutively expressed on the surface of immortalised HBECs but can become associated with the cell surface membrane after exposure to normal human plasma or recombinant C1QBP. C1QBP is not the major host receptor for IEs tested here but may act in combination with EPCR. Since primary HBEC constitutively expressed C1QBP, IE may have developed the capacity to interact with C1QBP to prevent the activation of the classical pathway of the complement system and thus ensures its survival and transmission.

II. Lay Summary

CM is characterised by the accumulation of IEs within the cerebral microvasculature, leading to vessel occlusion, and contributing to children becoming severely unwell, which can lead to death. Currently, there is no adjunctive therapy which can prevent the accumulation of IEs within children cerebral microvasculature. One strategy to reduce the number of children who die from malaria infection would be to develop an effective adjunctive therapy to prevent severe disease but not malaria infection.

The malaria parasite is capable of infecting and exploiting red blood cells, by modifying their surface to express highly variable adhesive proteins. The parasite uses these adhesive proteins to evade destruction via the spleen, by attaching IE to the lining of the blood vessels via diverse molecules expressed by human endothelial cells. This binding is called sequestration. Previous studies have identified a subset of parasite adhesive proteins involved in IEs sequestration. However, the major human endothelial molecules for these adhesive proteins remains a matter of debate but may include endothelial protein C receptor, and intercellular adhesion molecule 1. Previous works have proposed complement C1q binding protein as a potential endothelial molecule for IEs sequestration, but this adhesion phenotype has received less attention. In my research, I used an immortalised human endothelial cell and immunofluorescence assays to characterise the cellular localisation of complement C1q binding protein, and adhesion assays to determine the role of complement C1q binding protein in IE sequestration.

Firstly, I authenticated the endothelial nature of the immortalised human endothelial cell with markers including von Willebrand factor, platelet endothelial cell adhesion molecule 1, Dil-Acetylated- low density lipoprotein, and smooth muscle actin. As expected, I found that the immortalised endothelial cell showed positive intracytoplasmic staining for von Willebrand factor, Dil-Acetylated- low density lipoprotein, and positive surface staining for platelet endothelial cell adhesion molecule 1, and negative staining for smooth muscle actin. These results validate the endothelial nature of the immortalised human endothelial cell. Hence, I used the immortalised endothelial cell to investigate the cellular localisation of complement C1q binding protein. Unexpectedly, I observed that resting and activated (i.e, preincubation of the immortalised endothelial cell with TNF-α, a proinflammatory cytokine associated with severe malaria and it can increase/decrease the expression of some endothelial molecules) immortalised endothelial cell showed intracellular staining but not surface expression.

However, after incubation with soluble complement C1q binding protein or normal human plasma (which contains soluble complement C1q binding protein), the immortalised endothelial cell did display positive surface membrane expression of complement C1q binding protein. In addition, I found that primary endothelial cells constitutively expressed complement C1q binding protein but not immortalised endothelial cells. These results suggest that primary endothelial cells may express different surface receptors compared to immortalised endothelial cells.

Secondly, I assessed whether complement C1q binding protein plays an important role in IEs sequestration. I used four laboratory adapted parasite lines associated with severe and cerebral malaria in static adhesion assays with soluble proteins of endothelial molecules. I found that one parasite line binds to complement C1q binding protein, thrombospondin, and endothelial protein C receptor but the others did not bind to complement C1q binding protein. Therefore, I tested the ability of antibodies (an immune defence molecule) and soluble molecule of complement C1q binding protein and other soluble molecules to inhibit the interaction. I found that antibodies and soluble molecules of complement C1q binding protein did not prevent the interaction between IEs and the immortalised endothelial cell. However, antibodies and soluble molecules of endothelial protein C receptor did prevent the interaction. These data indicate that complement C1q binding protein is not the major endothelial molecule for IEs sequestration.

In summary, I have found that complement C1q binding protein is not constitutively expressed by the immortalised endothelial cell but it can become associated with the surface membrane of the immortalised endothelil cell when added exogenously or from normal human plasma. Complement C1q binding protein is not the major endothelial molecule for IE sequestration but it can act in association with endothelial protein C receptor. Because primary endothelial cells constitutively expressed complement C1q binding protein, IEs may have developed the ability to interact with complement C1q binding protein to prevent the activation of the classical pathway of the complement system and thus ensures its survival and transmission.

III. Dedication

I dedicate this thesis to my family. Thanks for your constant attention and support.

IV. Acknowledgements

I address my sincere gratitude to;

Professor Alexandra J Rowe, who supported me at every step along the way. She teaches me how to rigourously apply the scientific method to understand my research.

Dr Ian Maccormick, who arranged meetings, made valuable suggestions, encouraged me, and kept me on target.

My thesis committee, Joanne Thompson for their helpful guidance and advice.

The Darwin trust and the University of Edinburgh for the financial support and facilities to conduct my research.

The Rowe and Matthews lab, Florence Mclean, Molly Carlier, Stanley Otoboh, Brian Omondi, Jessie Jungels and Ellie Walker for your scientific feedback and exceptional human being.

Parasites & Microbes Research & Training Center in Bamako, Molecular Epidemiology and Drug Resistance Unit, Professor Abdoulaye Djimde for the connection and permanent support.

My friends and brothers, Apophys, Baig, Bath, Excellence, Grand-père, Hamzi, Klyde, La dichotomie, Macky, and Yogo for your listening and accompaniemnt.

My family.

V. Abbreviations

ATS	Acidic terminal segment
APC	Activated protein C
AKI	Acute kidney injury
AMA-1	Apical membrane antigen-1
BBB	Brain blood barrier
BSA	Bovine serum albumin
C1QBP	Complement C1q binding protein
CD62-E	E-selectin
CD62-P	P-selectin
CNS	Central Nervous System
CM	Cerebral malaria
CHO	Chinese hamster ovary
CSA	Chondroitin sulphate A
CSP	Circumsporozoite protein
CIDR	Cysteine-rich Interdomain Region
CIDR α1.4	Cysteine-rich interdomain region α 1.4
DAPI	4',6 diamidino-2-phenyllindole
Dil-Ac-LDL	Dil- Acetylated- low density lipoprotein
DCs	Domain cassettes
DBL	Duffy Binding-like
DMEM	Dulbecco's Modified Eagle's Medium
ECM	Endothelial cell medium
ELISA	Enzyme linked immunosorbent assay
EPCR	Endothelial Protein C Receptor
EtBr	Ethidium Bromide
FSC	Forward scatter
gC1qR	Globular head domain of C1q receptor
G6PD	Glucose 6 phosphate dehydrogenase
Ht	Haematocrit
HbAS	Haemoglobin AS
HbC	Haemoglobin C
HBECs	Human Brain Endothelial Cells
HSPGs	Heparan sulfate proteoglycans
HRP3	Histidine-rich protein
HRP2	Histidine-rich proteins
HBMEC	Primary human brain microvascular endothelial cell
HDMEC	Primary human dermal microvascular endothelial cell
HPMEC	Primary human lung microvascular endothelial cell
HRV	Human rhinoviruses

HUVECs	Human umbilical vein endothelial cells
HABP1	Hyaluronan binding protein 1
hCMEC/D3	Immortalised human brain endothelial cell
HBEC-5i	Immortalised human brain endothelial cells
ICAM1	Intercellular Adhesion Molecule1
IEs	Plasmodium falciparum infected erythrocytes
IFA	Immunofluorescence assay
IGF	Insulin-growth factor
IL10	Interleukin 10
LFA-1	Lymphocyte function-associated antigen 1
Mac-1	Macrophage-1 antigen
MRI	Magnetic resonance image
MFI	Median fluorescence intensity
NCAM	Neural Cell Adhesion Molecule
NO	Nitric oxide
NTS	N-terminal segment
OD	Optic density
PFA	Paraformaldehyde
PBS	Phosphate Buffered Saline
PE	Phycoerythrin
PfEMP1	Plasmodium falciparum erythrocyte membrane protein 1
PECAM1	Platelet Endothelial Cell Adhesion Molecule 1
PAR1	Protease-activated receptor-1
PC	Protein C
RDTs	Rapid diagnostic tests
RT	Room Temperature
RF	Rosette frequency
RPMI	Roswell Park Memorial Institute
SNBTS	Scottish National Blood Transfusion Service
SMAn	Severe malaria anaemia
SSC	Side scatter
SMA	Smooth muscle actin
sC1QBP	Soluble C1QBP
TSP	Thrombospondin
TRAP	Thrombospondin-related anonymous protein
TF	Tissue factor
TNF-α	Tumour necrosis factor-alpha
UM	Uncomplicated malaria
Ups	Upstream sequence
VCAM1	Vascular Cell Adhesion Molecule 1
vWF	Von Willebrand Factor
VV	Vybrant DyeCycle Violet
WHO	World Health Organisation

VI. Table of Contents

VII. List of Figures and Tables

1. CHAPTER I: Introduction

A general introduction to malaria and the thesis rationale is highlighted here. A specific introduction is also provided for each result chapters.

1.1. Malaria- a general background

The global burden of malaria was estimated at 263 million cases in 2023, mostly in sub-Saharan Africa (WHO, 2024). Malaria deaths were 597 000 worldwide, with 95% of deaths affecting young children in Africa (WHO, 2024). Malaria is a mosquito-borne disease caused by an *Apicomplexan* parasite belonging to the genus *Plasmodium*, of which *Plasmodium falciparum* is the deadliest species (WHO, 2024). In malaria-endemic areas, many of the children who die from malaria are infected with mature *P. falciparum* isolates that sequester (adhere to endothelial cells) densely in the brain microvasculature, and death is preceded by coma; this is recognised as CM (Molyneux, 1990).

In moderate and high malaria transmission settings, in the absence of clinical immunity, early diagnosis and treatment of malaria are required to prevent severe disease and death. Antigen-based rapid diagnostic tests (RDTs) for *P. falciparum* targeting the histidine-rich proteins 2 and 3 (HRP2 and HRP3) are widely deployed to establish an accurate diagnosis where the gold standard blood-film examination by light microscopy is not available (WHO, 2024). Injectable artesunate is the standard of care for the treatment of CM and is highly efficacious at killing parasites (Dondorp et al., 2010; Kremsner et al., 2016). Despite this efficacy, 10-25% of children affected by CM will die (Dondorp et al., 2010; Seydel et al., 2015). However, among survivors at hospital discharge 41% will exhibit abnormal neurologic examinations. Having more than 4 seizures during admission has been strongly associated with neurocognitive and learning disabilities among children less than 5 years of age (Clark et al., 2023). These adverse outcomes may occur because intravenous antimalarial drugs kill circulating parasites, but they do not immediately reverse sequestration (Hughes et al., 2010).

Hence, the ability of mature stages of IE to sequester in HBECs (MacPherson et al., 1985; Taylor et al., 2004) constitutes a threat for case management of patients with this life-threatening disease. To mitigate this risk, it is imperative to better understand the pathophysiology of CM and identify interventions to improve its clinical outcome. Other

current threats to effective management of malaria include deletions of the *Pfhrp2* and *Pfhrp3* genes in IE isolates resulting in false-negative diagnostic tests and the spread of artemisinin resistance in Asian and African countries (Ashley et al., 2014; Balikagala et al., 2021; Uwimana et al., 2020).

In this chapter, I summarise knowledge about severe malaria epidemiology, clinical presentations of severe malaria, pathophysiology, immune protection mechanisms, the molecular basis and current approaches used to study IE cytoadhesion. I also state the rationale and aims of the thesis.

1.2. The epidemiology of severe malaria

In areas of intense and moderate transmission, children in the first 1-5 years of life are the most affected group, whereas in areas of low transmission, the disease affects all age groups (White, 2018). In all endemic areas, infection and clinical disease are infrequent in children up to age 6 months due to passive immunity from maternal antibodies (Riley et al., 2001). The disease presentations also differ with changes in the status of transmission, in areas of high transmission, anaemia in young children is the main manifestation of severe malaria. In areas with intermediate transmission, coma and metabolic acidosis in a wider age range of children are the main complications observed. In places where the disease transmission is lowest, all individuals are at risk of developing severe malaria (Snow et al., 1997) (Figure 1-1). Jaundice and pulmonary oedema are common manifestations in adults, whereas anaemia, hypoglycaemia and convulsions predominate in children. Moreover, recent work suggests that acute kidney injury (AKI) is also common in children with severe malaria, and this was unrecognised previously due to the use of inappropriate diagnostic test thresholds (Conroy et al., 2023). Consequently, some African children with severe malaria experience an increase level of uric acid associated with mortality and cognitive impairment (Bond et al., 2025). CM and metabolic acidosis may occur at all age categories (Marsh et al., 1995; Marsh & Snow, 1997; Riganti et al., 1990). However, in region of intense transmission, adults continue to carry malaria parasites, but the frequency of clinical disease is extremely reduced. This tolerance of clinical disease in adults, known as clinical immunity, usually is never reached in low transmission settings (Miller et al., 1994).

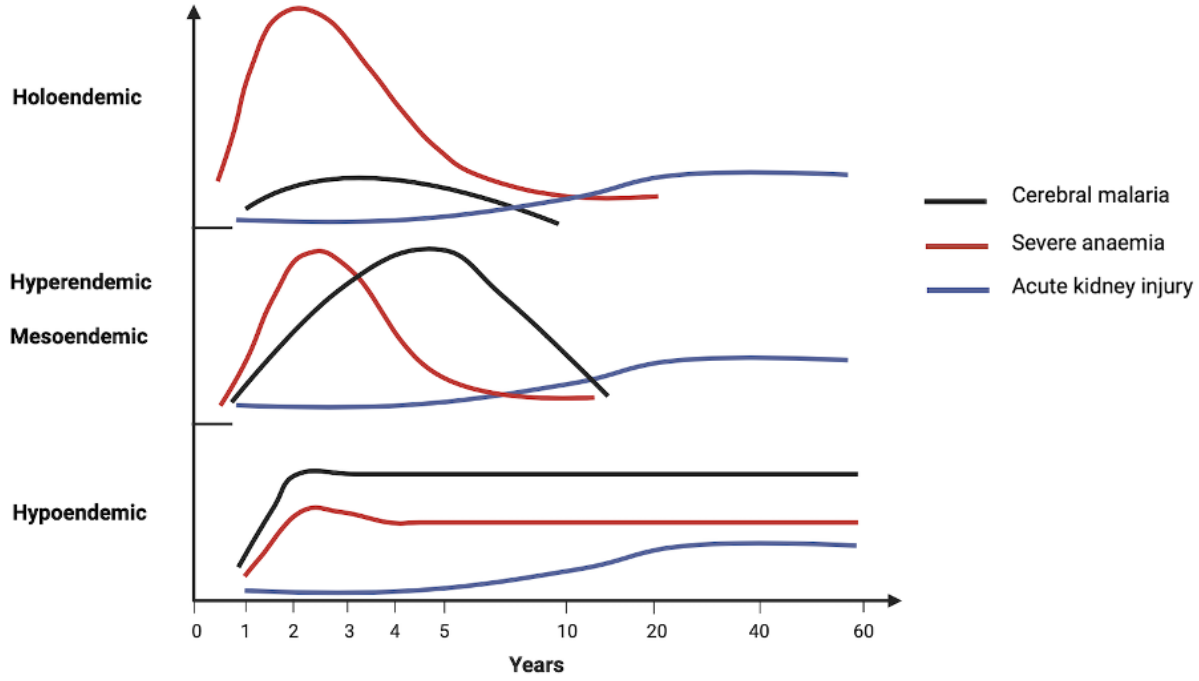


Figure 1-1. Illustration of the relationship of severe falciparum malaria intensity to age at different levels of malaria transmission Parasite rate or spleen rate has been used to define levels of malaria endemicity in children aged 2-9 years, Holoendemic: constantly ≥ 75% with a low adult spleen rate, parasite density decreases rapidly between 2 and 5 years of age; hyperendemic: constantly ≥ 50%; mesoendemic: 10- 50% and hypoendemic: 0- 10% (WHO, 2021). A recent study showed that renal injury in also common in children with severe malaria (Conroy et al, 2023). The figure is adapted from White, 2018 **(White, 2018)**.

1.3. Life cycle of *Plasmodium falciparum*

P. falciparum undergoes multiple development stages in both the human and mosquito hosts (Figure 1-2). Following an infectious bite from an *Anopheles* mosquito, sporozoites migrate into blood vessels and circulate in the blood until they infect liver cells. This interaction is mediated by parasite circumsporozoite protein (CSP) and hepatocyte heparan sulfate proteoglycans (HSPGs) (Herrera et al., 2015), with the contribution of thrombospondin-related anonymous protein (TRAP) and apical membrane antigen-1 (AMA-1) released from the parasite apical organelles (micronemes and rhoptries) (Cowman et al., 2016).

In the hepatocyte, sporozoites replicate asexually for 5-10 days to form exo-erythrocytic merozoites, and maturation is concluded by hepatocyte rupture, releasing up to 40,000 membrane bound invasive merozoites (merosomes) into the bloodstream (Sturm et al., 2006). Invasive merozoites rapidly colonise erythrocytes to escape the host immune responses using a stepwise process including pre-invasion, active invasion and echinocytosis (Weiss et al.,

2015). Merozoites develop consecutively into ring-stage, early and late pigmented trophozoite-stage and schizont-stage within the IEs. Pigmented trophozoite and schizont stages of *P. falciparum* exhibit adhesion molecules on the surface of their IEs. These ligands allow the mature stages of *P. falciparum* to adhere to the microvasculature of vital organs and lead to their sequestration (MacPherson et al., 1985; Taylor et al., 2004; Tembo et al., 2014). The clinical manifestation of malaria occurs during this erythrocytic phase. Parasites replicate asexually inside the erythrocytes for 48 hours and the cycle is completed by bursting of the IEs and release of 16-32 newly formed daughter merozoites into the blood, which can each invade a new erythrocyte (Cowman et al., 2016). Upon each new round of the erythrocytic cycle, parasites must choose between two developmental pathways, to continue to replicate asexually and invade new erythrocytes, or, to commit to development into a sexual form, called gametocytes, which differentiate into male and female forms. When mature gametocytes are picked up by mosquitoes during a blood meal, they mate inside the mosquito gut to form a zygote, which then undergoes meiosis and transforms into an ookinete that traverses the mosquito midgut wall to form an oocyst. Continued rounds of asexual replication within the oocyst forms sporozoites, which then move to the salivary glands and are released back into a new human host when the mosquito takes a blood meal (Figure 1-2) (Cowman et al., 2016).

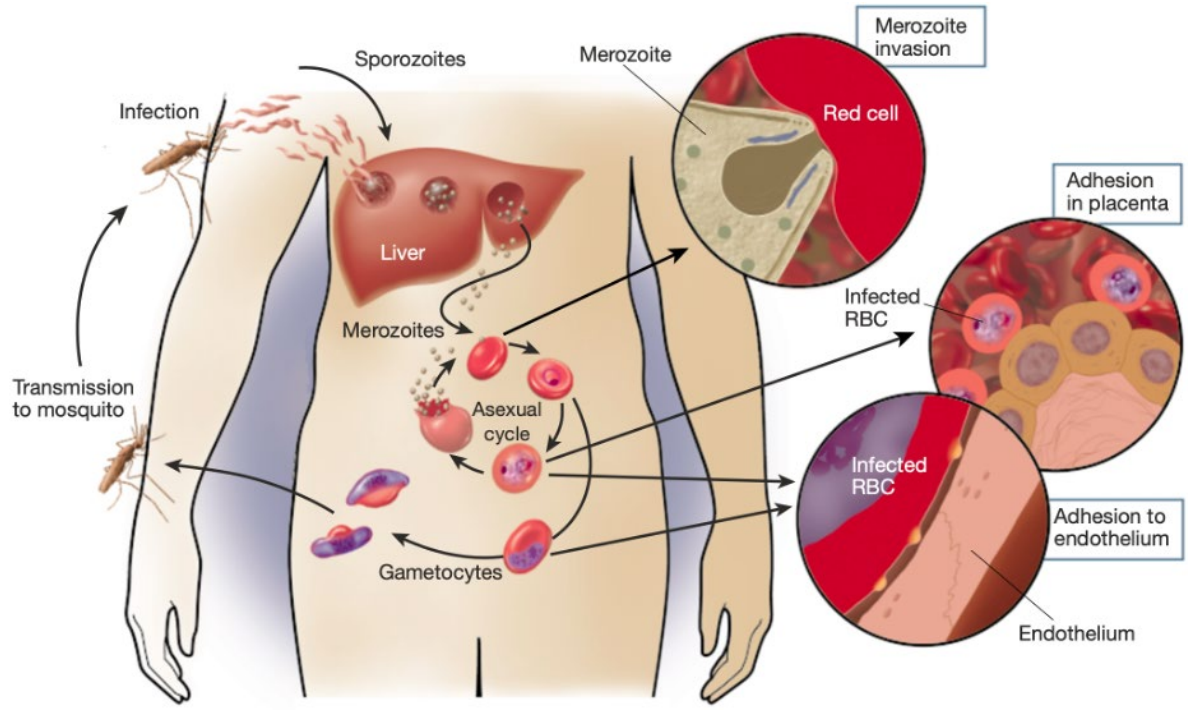


Figure 1-2. Life cycle and pathogenesis of falciparum malaria. An illustration of the developmental specific stages (from infection to transmission) and sequestration sites of Plasmodium falciparum. The cellular events and molecular interaction determine the disease status. All human Plasmodium spp. invade erythrocytes by the same

mechanism, but only mature P. falciparum infected erythrocytes (IEs) adhere to endothelium, placenta and avoid spleen dependent clearance mechanisms. The figure is taken directly from Miller et al 2002 (Miller et al., 2002).

1.4. Clinical features of *P. falciparum* malaria

The clinical spectrum of *P. falciparum* infection ranges from asymptomatic to uncomplicated malaria (UM), in which a subset of cases results in severe life-threatening complications such as severe malaria anemia (SMAn), respiratory distress and CM (Miller et al., 2002). Asymptomatic infection is characterised by the detection of malaria parasites in the peripheral blood of a patient without any clinical symptoms of the disease. The clinical features of malaria occur during the erythrocytic stage of the infection, when the parasite grows and multiplies in cyclic manner within the host erythrocytes (Figure 1-2). Patients with malaria present symptoms including fever, chills, headache, malaise, vomiting and mild diarrhoea. These symptoms are representative of UM (White, 1996). However, severe malaria in African children is classified into three major clinical types: SMAn, CM, and respiratory distress. All three types, whether occurring alone or in combination, can kill rapidly or resolve quickly after appropriate treatment (Newton et al., 1998). For research and clinical management purposes, severe malaria is defined by the presence of an asexual parasitaemia and one of the following clinical criteria: CM, altered consciousness (Blantyre Coma Score ≤ 2) at least 1 hour after termination of seizures and exclusion of other causes of encephalopathy; SMAn (packed cell volume < 15% or hemoglobin < 5g/dL); acidosis or respiratory distress (lactate > 5 mM and/ or chest in-drawing or deep breathing); prostration (inability to sit or breastfeed in children old enough to do so based on their age); hypoglycemia (blood glucose < 2.2 mM); multiple seizures (≥ 2 convulsions in the preceding 24 hours); repeated vomiting or dehydration (≥ 3 vomiting or inability to take oral medication) and hyperparasitaemia (more than 10% of erythrocytes infected) (WHO, 2000).

1.5. Pathophysiological mechanisms of severe malaria

The key pathophysiological process underlying CM remains elusive and a constellation of clinical, and pathological findings have been suggested such as the reduce deformability of uninfected erythrocytes during severe malaria, destruction of IEs and release of parasite toxins into the systemic area, interaction between IEs and uninfected erythrocytes, host immune responses and the sequestration of IEs in human microvasculature. Some or all these

mechanisms may combine to play a critical role in the pathogenesis of severe malaria (Milner et al., 2014; Taylor et al., 2004; WHO, 2000).

1.5.1. Risk factors associated with severe malaria

According to the World Health Organisation (WHO) in the absence of severe disease signs, a child with a parasitaemia over 10% of the total body erythrocytes should be considered as severe malaria case (WHO, 2014). The relation between the peripheral malaria parasitaemia and severe disease presentation in different populations and age groups remains controversial. CM in African children has showed a poor correlation between the density of peripheral parasitaemia and the depth of coma (Molyneux, 1990). While postmortem studies have demonstrated the presence of higher number of IE in the brain (Bernabeu et al., 2016) and other vital organs of adults affected by CM (Ponsford et al., 2012). However, in Tanzania a birth cohort of children experiencing severe malaria has shown that higher parasitaemia is associated with CM but alone is not sufficient to explain the mechanism of CM (Goncalves et al., 2014). The peripheral asexual stage parasitaemia did not always reflect the burden of mature *P. falciparum* that sequestered in vital organs.

Plasma *Pf*HRP2 has been proposed as an alternative to better estimate the total parasite load within each tissue (Dondorp et al., 2005). HRP2 is a protein produced by *P. falciparum* and secreted into the circulation at schizont rupture, thereby the quantity of *Pf*HRP2 released is proportional to the level of schizogony. Subsequent study conducted in high transmission setting have found that *Pf*HRP2 level is not correlated to severe disease in Papua New Guinea children (Manning et al., 2011). However, later studies performed in different epidemiological settings argue that *Pf*HRP2 concentrations are associated with disease severity (Hendriksen et al., 2013), and mortality in Tanzanian children (Rubach et al., 2012). A multicentric study in Africa has found an association between *Pf*HRP2 concentration and death in paediatric population but not with the peripheral blood parasitaemia (Hendriksen et al., 2012). Using data from 2649 paediatric and adults patients from four independent studies, the combination of platelet count of ≤150,000/μL and a plasma P*f*HRP2 concentration of more than ≥1000 ng/mL provided a good quantitative parameters to diagnose severe malaria (Watson et al., 2022). The use of P*f*HRP2 level alone to diagnose severe malaria in high transmission setting remains a

matter of debate but the combination with platelet count can improve the diagnosis (Watson et al., 2022).

Therefore, other studies have proposed examining patients for malaria retinopathy (ocular fundus) to improve the diagnosis of CM (Lewallen et al., 2000; Taylor et al., 2004). WHO recognised retinopathy positive malaria by the presence of retinal haemorraghes, classicaly white-centred haemorraghes, or any retinal whitening, or any vessel colour change (orange or white) in a patient with clinical CM (WHO, 2014). Other investigators supported the use of malaria retinopathy to diagnose and study CM and thereby avoid incorrect inclusion of children with incidental parasitaemia and coma due to other causes. They also underlined the use of plasma P*f*HRP2 level and retinopathy positive for a better diagnosis (Kariuki et al., 2014; MacCormick et al., 2014). Despite compelling approaches to discriminate CM from other causes of severe diseases using retinopathy criteria, a study pointed out that plasma P*f*HRP2 concentrations may be more relevant to correctly diagnose CM than retinopathy positive (Park et al., 2017). A more recent study has revealed that platelets and white blood cells counts associated with retinopathy can substantially improve the diagnosis of CM (Lin et al., 2023). Additionally, brain swelling detected by magnetic resonance image (MRI) has been associated with increased risk of death in children with retinopathy positive CM in Malawi (Seydel et al., 2015). However, the ocular fundus examination and MRI necessitate well-trained investigators, and more investment to acquire and maintain the MRI. This can be a challenge in resources limited settings where the disease is prevalent. Hence, another research group has proposed a combination of P*f*HRP2 level, group A *var* gene transcript (which encodes severe malaria-associated *Plasmodium falciparum* erythrocyte membrane protein 1 (PfEMP1) adhesion molecules), and lower platelet level as the best biological predictor to discriminate severe malaria from other causes of severe illness (Sahu et al., 2021). In clinical practices, CM signs, retinopathy positivity, and lower platelet level can be used at bedside to start CM cases management and improve clinical outcome of the disease.

1.5.2. Destruction of erythrocytes

SMAn is one of the deadliest complications in children infected with IEs. Diverse pathogenic mechanisms have been proposed to explain the reduction of circulating erythrocyte numbers during malaria infection. The massive destruction of infected and uninfected erythrocytes,

insufficient erythropoiesis and bone marrow dyserythropoiesis have been incriminated to play a role in the pathophysiological processes of SMAn (White, 2018). Other factors associated with SMAn are vitamin A and B12 deficiencies, infectious diseases such as HIV, Epstein-Barr virus, bacterial infections, hookworm, and host polymorphisms like G6PD deficiency (glucose 6 phosphate dehydrogenase) and single-nucleotide polymorphisms in the promoter region of the gene encoding IL10 (Interleukin 10) (Calis et al., 2016).

Plasmodium spp. are obligatory intraerythrocytic parasites, therefore there is a destruction of erythrocytes containing mature parasites at schizont rupture (Figure 1-2). In severe falciparum malaria, high parasite burden is a risk factor for developing anaemia, but there is also accelerated destruction of uninfected erythrocytes resulting in their shortened life span compared to healthy patients and those with UM (Jakeman et al., 1999; Looareesuwan et al., 1991). The plausible explanation for the decline in haematocrit concentration is the haemolysis of infected and uninfected erythrocytes due to a complement-mediated immune response (Woodruff et al., 1979). The surface of erythrocytes of children experiencing SMAn is modified by the deposition of IgG (Immunoglobulin G) and alterations in levels of complement regulatory proteins (Waitumbi et al., 2000). Another reason is the absorbtion of parasite pigment (haemozoin) onto the surface of uninfected erythrocytes, where it fixes complement and immunoglobulins such as IgG and IgM, and erythrocytes targeted by this immune process are cleared in the spleen. This destruction contributes to the splenomegaly caused by phagocytosis of infected and uninfected erythrocytes (Woodruff et al., 1979). Additionally, the destruction of infected and uninfected erythrocytes during severe malaria may be linked with oxidative damage (Casals-Pascual et al., 2006; Greve et al., 2000) and reduced α-tocopherol reserve in the erythrocyte membrane, highlighting that local antioxidant depletion may contribute to erythrocyte loss (Griffiths et al., 2001).

Dyserythropoiesis or ineffective erythropoiesis leading to SMAn is associated with intramedullary deposition of malaria pigment in the bone marrow. Postmortem examination of the bone marrow of African children who have died from malaria showed that pigmented erythroid and myeloid cells are associated with abnormal development of erythroid precursors (Casals-Pascual et al., 2006), and increased apoptosis of erythroid progenitor cells *in vitro,* independently of inflammatory mediators (Lamikanra et al., 2009). However, other investigators using an *in vitro* approach have demonstrated that the accumulation of *P.*

falciparum haemozoin in the bone marrow inhibits erythropoiesis by acting both directly on erythroid cells, and indirectly through inflammatory mediators such as TNF-α (tumour necrosis factor-alpha) and nitric oxide (NO) produced by haemozoin-stimulated macrophages (Awandare et al., 2011). Additional evidence describes independent pathways used by haemozoin and TNF-α to inhibit erythropoiesis (Lamikanra et al., 2015).

More recent works have described the ability of IE to secrete extracellular vesicles that alter the membrane of uninfected erythrocytes, facilitate their invasion and malaria parasite growth (Dekel et al, 2021). The evaluation of plasma biomarkers in children with SMAn revealed that higher 20S proteasome and lower insulin-growth factor (IGF) are associated with anaemia and altered erythrocyte membrane properties (Mahamar et al, 2022). The 20S proteasome may play a role in modifying the stiffness of erythrocyte membrane by targeting four phosphorylated cytoskeletal proteins β-adducin, ankyrin-1, dematin and erythrocyte membrane protein band 4.1 (Dekel et al, 2021). While IGF, which is involved in erythroid maturation, might also contribute to ineffective erythropoiesis (Mahamar et al, 2022).

1.5.3. Sequestration of infected erythrocytes

Accumulation of IEs within the human brain microvasculature is the pathological hallmark of CM and it provides a plausible pathophysiological explanation for coma, related to microvascular obstruction and hypoxia. Postmortem studies from patients dying with *P. falciparum* infections have described cerebral venules and capillaries filled with mature IEs, while cerebral sequestration is much sparser/absent in UM (MacPherson et al., 1985; Taylor et al., 2004). Sequestration is caused by specific cytoadhesion interactions between parasite-derived adhesion proteins exposed on the surface of IEs and host endothelial cell receptors exhibited on the surface membrane of HBECs such as EPCR (Turner et al., 2013) and ICAM1 (Berendt et al., 1989). These host-parasite adhesion interactions are described more fully in section 1.10.4.

Cytoadhesion can be substantially increased when adherent IEs bind to two or more uninfected erythrocytes, this process is called rosetting (Rowe et al., 1997) or platelets, this is recognised as platelet-mediated clumping (Pain et al., 2001). Rosetting is a parasite phenotype associated with severe disease. In postmortem studies, rosette-like cluster of cells have been observed in the microvasculature of fatal malaria cases (Barrera et al., 2018; Dondorp et al., 2004). *Ex vivo*

studies have also shown that rosettes occurred more commonly with parasites obtained from patients with CM than patients experiencing UM (Carlson et al., 1990; Doumbo et al., 2009; Rowe et al., 1995; Treutiger et al., 1992). Both cytoadherence (Rowe et al., 2009) and rosetting (Ghumra et al., 2012; Juillerat et al., 2011; McLean et al., 2025; Rowe et al., 1997) are mediated by the highly polymorphic protein PfEMP1 encoded by the *var* gene family. *In vitro* studies have also demonstrated the ability of culture-adapted IEs expressing a rosette-mediating PfEMP1 variant to cytoadhere to HBEC receptors after rosette disruption (Adams et al., 2014; McLean et al., 2025). These results suggest the development of interventions targeting this dual binding phenotype.

Platelet-mediated clumping is another *P. falciparum* cytoadherence phenotype. The formation of clumps due to the interaction between laboratory lines of *P. falciparum* and platelets requires the expression of the platelet surface glycoprotein CD36 (Pain et al., 2001), with some evidences suggesting that P-selectin/CD62-P (Wassmer et al., 2008) and C1QBP (Biswas et al., 2007) on platelets can also be considered as receptors for clumping. Using clinical samples, *in vitro* platelet- mediated clumping has been associated with severe malaria (Mayor et al., 2011). Additionally, an *in vitro* study showed that platelets can act as bridges to mediate IE binding to CD36-deficient HBECs (Wassmer et al., 2004), while a postmortem study revealed that platelet accumulation in the brain was significantly higher in CM patients than in those with non-cerebral malaria (Grau et al., 2003). However, another study suggested that clumping is related to high parasitaemia but not disease severity (Arman et al., 2007). The association between platelet-mediated clumping and disease severity remains controversial. Further studies are needed to understand the role of platelet-mediated clumping in CM.

Sequestration of IEs with uninfected erythrocytes and platelets within the cerebral vessels impedes microvascular blood flow (Dondorp et al., 2008; Hanson et al., 2012; Hanson et al., 2015; O'Brien et al., 2022). Furthermore, mature IEs become progressively less deformable so that they have more difficulty transiting through the microvasculature. Studies of Thai adults (Dondorp et al., 1999; Dondorp et al., 1997), and Kenyan children (Dondorp et al., 2002) have shown strong association between reduced erythrocyte deformability and fatal malaria outcome. However, in Kenyan children reduced erythrocyte deformability can be restored by blood transfusion (Dondorp et al., 2002). Major consequences of IE cytoadhesion during CM are reduction in delivery of oxygen to the brain and the development of cerebral hypoxia leading to anaerobic glycolysis and death (Dondorp et al., 2008; Warrell et al., 1988).

1.6. Coagulopathy

Adherent IEs have been shown to induce tissue factor (TF) production by endothelial cells *in vitro* and stimulate the extrinsic coagulation pathway (Francischetti et al., 2007). The secreted parasite product *Pf*HRP2 also contributes to a procoagulant environment by blocking the activity of antithrombin III (Ndonwi et al., 2011). Additionally, the parasite's EPCR binding ligand, cysteine-rich interdomain region α 1.4 (CIDR α1.4) domain of PfEMP1, prevents the generation of activated protein C (APC) (Gillrie et al., 2015), the major anticoagulant protein. These *in vitro* observations and the demonstration of fibrin deposition in more than 85% of cerebral microvessels in children who died from CM (Moxon et al., 2013) corroborate the potential pathological significance of microvascular thrombosis in paediatric CM.

An important molecule between the malaria parasite and the human coagulation system is thrombin, which coordinates the balance between an anticoagulant state by the activation of protein C and a procoagulant state by fibrin formation (Mosnier et al., 2007). Binding of thrombomodulin (a glycoprotein constitutively expressed on the surface of human endothelial cells) to the thrombin exosites I and II forms the complex thrombin-thrombomodulin. This complex interacts with the protein C- EPCR complex and triggers the activation of protein C. APC in association with the protease-activated receptor-1 (PAR1) provides a cytoprotective effect on endothelial barrier function (Figure 1-3). However, in the presence of IEs cytoadhering to EPCR, the direct interaction between thrombin exosite I and PAR1 can induce proinflammatory effects, increasing endothelial cell apoptosis and blood brain barrier dysfunction (Figure 1-3) (Gillrie et al., 2016).

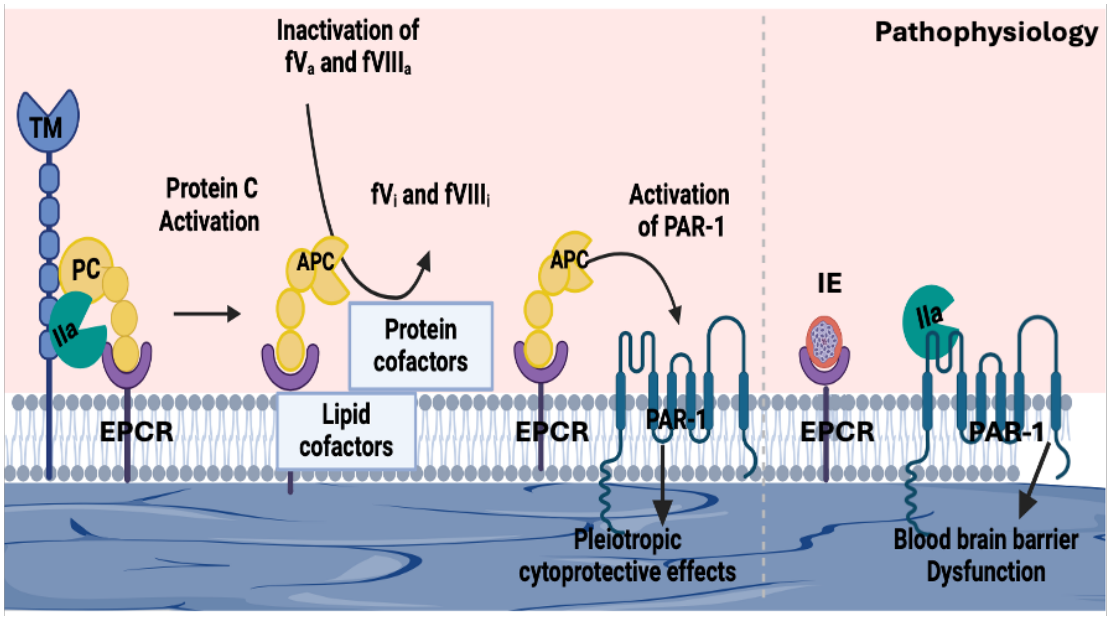


Figure 1-3. Illustration of EPCR role in protein C activation and severe malaria pathophysiology. Protein C (PC) activation involves the endothelial cell receptor, EPCR, and the complex thrombomodulin (TM)- thrombin (IIa). Binding of PC to EPCR induces the activation of protein C (APC). PC and APC have the same affinity to EPCR. APC exerts its anticoagulant activity when bound to the endothelial cell membrane and cooperates with diverse lipoproteins (Protein and lipid cofactors). As an anticoagulant, APC cleaves the activated cofactors Va (fVa) and VIIIa (fVIIIa) to generate the inactivated cofactors fVi and fVIIIi. Physiological activities of APC involve its interaction with EPCR and protease-associated receptor-1 (PAR-1). These activities include APC-mediated alteration of gene expression, anti-inflammatory activities, anti-apoptotic activities, and protection of endothelial barrier function. Together, these activities referred to as APC's cytoprotective effects. During cerebral malaria, *P. falciparum* infected erythrocytes (IE) bind to EPCR and sequester in the brain microvasculature. This interaction compromises the secretion of APC and facilitates the adhesion between IIa and PAR-1. Collectively, these interactions lead to inflammation, coagulation and blood brain barrier disruption. The figure is adapted from (Mosnier et al., 2007).

1.7. Host inflammatory responses

The plasma concentration of TNF-α, a proinflammatory cytokine, is elevated in acute malaria and is particularly high in children with profound coma (Kwiatkowski, 1990). However, TNF-α concentrations in uncomplicated *Plasmodium vivax* infections are as high as those found in CM patients (Karunaweera et al., 1992). Therefore, if TNF plays a role in CM pathogenesis, it may be at local tissue level, where the schizogony of a mature IE occurs and the parasite toxins that induce TNF production are released. For instance, TNF has been shown to upregulate the expression of ICAM1 (Weksler et al., 2005; Xiao et al., 1996; Zougbede et al., 2011) a host endothelial cell receptor involved in IE cytoadhesion and sequestration, and also TNF and other

cytokines increase cytoadhesion of IE *in vitro* (Xiao et al., 1996; Zougbede et al., 2011). It is therefore possible that TNF and other proinflammatory cytokines contribute to malaria severity by promoting IE cytoadhesion and sequestration.

1.8. Host erythrocyte polymorphisms and severe malaria

The importance of cytoadhesion in severe malaria is supported further by the increased prevalence of malaria-protective point mutations in the haemoglobin gene within human populations living in areas where malaria is endemic: e.g., haemoglobin C (HbC) and haemoglobin AS (HbAS). The mechanism of protection against severe malaria of these haemoglobinopathies is due, at least in part, to abnormal display of PfEMP1 on the surface of IEs, which reduces their ability to adhere to endothelial cells (Fairhurst et al., 2005; Fairhurst et al., 2012; Petersen et al., 2021; Sanchez et al., 2019) and to form rosettes (Opi et al., 2014) . Furthermore*, P. falciparum* rosetting is also reduced in blood group O erythrocytes compared with group A, B, and AB, providing an explanation for the malaria protective effect of blood group O (Opi et al., 2023; Rowe et al., 1995; Rowe et al., 2007). These observations clearly support the hypothesis that reducing cytoadherence and rosetting decreases the risk of developing severe malaria. Hence, reduction or reversal of parasite adhesion interactions with host cells can be considered a plausible therapeutic option for improving clinical outcomes.

1.9. Immunity to severe malaria

Placental malaria is a good model for future studies on immunity to severe malaria. Women during their first pregnancy are vulnerable to malaria infection of their placenta leading to anaemia in women and low birthweight in newborn babies (Desai et al., 2007). However, immunity develops quickly during consecutive pregnancies. This immunity is mediated by antibodies that inhibit the interaction between the conserved PfEMP1 variant VAR2CSA (a member of PfEMP1 variant expressed on the surface of IEs) and the placental receptor chondroitin sulphate A (CSA). The anti-VAR2CSA antibodies block adhesion of geographically diverse parasites in a strain-independent manner (Fried et al., 1998). This result paves the way for development of an effective intervention against pregnancy-associated malaria (Gamain et al., 2021). If similar global strain-transcending adhesion-blocking antibodies are demonstrated against PfEMP1 variants associated with severe paediatric

malaria, this would provide strong rationale for similar development of an intervention to reduce malaria deaths in children.

Antibodies to the IE surface (most likely to PfEMP1) can prevent the interaction between IEs and HBECs (Reyes et al., 2024; Udeinya et al., 1983). PfEMP1 diversity and antigenic variation may help to explain the slow and gradual development of clinical immunity in areas of intense transmission (Griffin et al., 2015; Obeng-Adjei et al., 2020; Tessema et al., 2019). It has been suggested that the first malaria infections in infants and young children are dominated by parasites expressing group A and B/A PfEMP1s (described in more detail in section 1.10 below) (Cham et al., 2010; Cham et al., 2009). Then, infants and children acquire immunity (e.g. antibodies) to these virulence-associated PfEMP1 types after a few infections (Goncalves et al., 2014; Gupta et al., 1999) and immunity to severe disease is essentially complete by the age of five years. Furthermore, the fact that significant immunity to severe malaria develops after a relative low number of infections, suggests that some component of immunity can transcend the difference between parasites isolates. This could be, at least in part, related to the acquisition of strain-transcending antibodies to PfEMP1 (Ghumra et al., 2012; McLean et al., 2024). A more recent study also claims the discovery of broadly reactive inhibitory monoclonal antibodies to CIDRα1, the PfEMP1 domain that interacts with human EPCR (Reyes et al., 2024) . However, the Reyes et al. study focuses on PfEMP1 recombinant proteins instead of IEs expressing native PfEMP1 proteins. Therefore, it remains crucial to assess the ability of these antibodies to inhibit and reverse the binding of heterologous laboratory-adapted parasite lines and freshly collected field isolates from CM patients to HBEC.

1.10. Molecular basis of cytoadhesion

The cytoadhesion of IE to HBECs plays an important role in the occurrence of CM. Sequestration happens due to the molecular interaction between parasite adhesion ligands expressed on the surface of IE and host receptors exposed on the surface of HBECs (Rowe et al., 2009). The use of *in vitro* panning assays (to enrich *P. falciparum* strains for adhesion to HBEC) allows selection of IEs to become more adhesive to HBECs (Claessens & Rowe, 2012). This has enabled the identification of DC8 and DC13 containing PfEMP1 variants as major parasite HBEC-binding molecules linked to severe malaria (Avril et al., 2012; Claessens et al., 2012). The selected IE can also facilitate the identification of host endothelial proteins that are putative receptors to which they bind (Turner et al., 2013). An effective intervention against CM could potentially be developed but our current understanding remains incomplete.

1.10.1. PfEMP1 structure and classification

PfEMP1 is a highly polymorphic protein, encoded by the *var* gene family, that is involved in binding interactions between IEs and host cells (Baruch et al., 1995; Smith et al., 1995; Su et al., 1995). Each parasite genome encodes approximately 60 different variants of PfEMP1 (Gardner et al., 2002; Kraemer & Smith, 2003) and by mutually exclusive expression, only one *var* gene is transcribed per cell (Voss et al., 2006; Zhang et al., 2022). *Var* genes are encoded in two exons. The first exon encodes the diverse extracellular binding region and the transmembrane domain (Figure 1-4). The second exon is more conserved and encodes the acidic terminal segment (ATS) domain that is intracellular and postulated to anchor PfEMP1 at knobs (Mayer et al., 2012).

PfEMP1 extracellular domains comprise: the N-terminal segment (NTS) located at the amino terminus of all PfEMP1, the Duffy Binding-like (DBL) domains and the Cysteine-rich Interdomain Region (CIDR). DBL domains have seven sequence classes (α, β, γ, δ, ζ, ε and pam) and CIDR domains possess five distinct sequence classes (α, β, γ, δ and pam) (Figure 1-4). Consensus motifs are described for the different DBL and CIDR types. These sequence classes can be divided further into numerical subclasses (Rask et al., 2010). While the total number of DBL and CIDR domains varies between different PfEMP1 variants, PfEMP1 domain architecture is not randomly arranged, because certain tandem domain associations are commonly seen, such as DBLαCIDRα and DBLδCIDRβ/γ (Otto et al., 2019; Rask et al., 2010; Smith et al., 2000; Su et al., 1995).

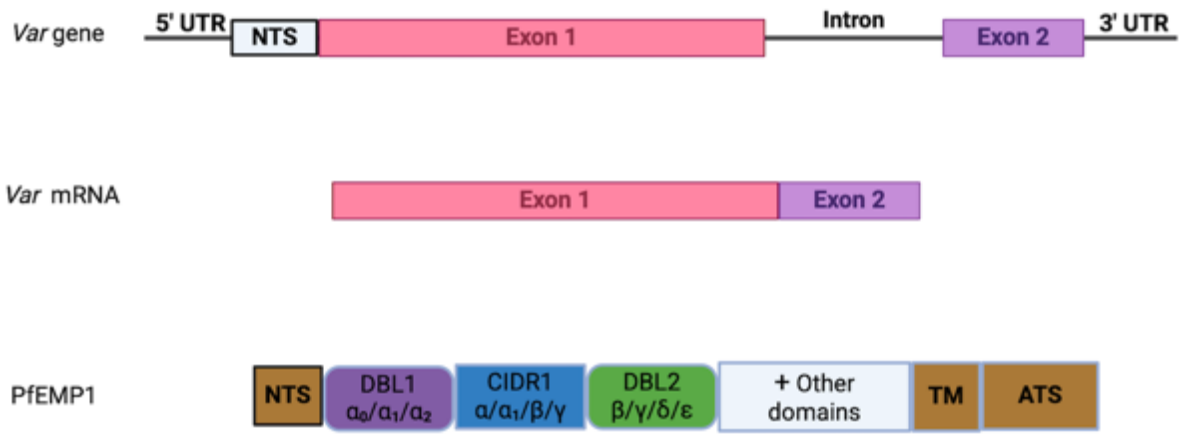


Figure 1-4. Illustration of a *var* gene encoding *P. falciparum* erythrocyte membrane protein 1(PfEMP1). The gene is composed of two exons, exon 1 in red and exon 2 in purple, and one intron. The main types of PfEMP1 head structure and domains are indicated. NTS = N-terminal segment; DBL = Duffy binding like; CIDR = Cysteine-rich interdomain region; TM = Transmembrane domain; ATS = Acidic terminal segment.

Var genes can be classified into three major groups (group A, B and C) based on chromosome location, transcriptional orientation and the upstream sequence (Ups). All UpsA (group A) and most UpsB (group B) *var* genes are found in the chromosomal sub-telomeric regions. UpsC (group C) *var* genes are found towards the centre of chromosomes (Gardner et al., 2002; Kraemer & Smith, 2003), while two intermediate groups, B/A and B/C, represent transitions between the three major groups (Lavstsen et al., 2003). Later classification of PfEMP1 based on tandem sets of domain subclasses that occur together in multiple parasite genomes, named domain cassettes (DCs), may represent functionally conserved units (Rask et al., 2010).

1.10.2. PfEMP1 and disease phenotypes

The differential expression of PfEMP1 groups is linked to disease phenotype. IE selected *in vitro* for adhesion phenotypes associated with paediatric children severe malaria preferentially expresses PfEMP1 encoded by group A and B/A *var* genes (Jensen et al., 2004). Evidence from naturally infected children from endemic areas shows that parasites causing severe disease express high levels of group A and B/A *var* genes, whereas those causing UM mainly express group B and C *var* genes (Kyriacou et al., 2006; Warimwe et al., 2012). Additionally, IE that bind to uninfected erythrocytes to form rosettes express predominantly group A *var* genes (Ghumra et al., 2012; McLean et al., 2025; Rowe et al., 1997; Vigan-Womas et al., 2012). Structural and functional grouping of PfEMP1 into DCs has enabled researchers to link IE expressing DC8 and DC13 with severe and CM (Avril et al., 2012; Claessens et al., 2012; Lavstsen et al., 2012). Recently, PfEMP1 DC11, DC15 and DC8 have been shown to mediate rosetting (McLean et al., 2025) and are associated with severe malaria (Duffy et al., 2019; Tonkin-Hill et al., 2018).

1.10.3. PfEMP1 binding domains

Previous work showed that DC8 and DC13 containing PfEMP1 variants are expressed by parasites selected on HBECs and are associated with severe malaria including SMAn and CM

(Avril et al., 2012; Claessens et al., 2012; Lavstsen et al., 2012). DC8 consists of 4 domains (DBLα2- CIDRα1.1- DBLβ12-DBLγ4/6) and DC13 is composed of 2 domains (DBLα1.7-CIDRα1.4) (Figure 1-5). DC8 and DC13 expressing parasite lines interact with diverse cerebral and non-cerebral endothelial cells (Avril et al., 2013; Ortolan et al., 2022). A screening approach using an array of human plasma membrane proteins showed that the CIDRα1 domain in DC8, DC13 and a subpopulation of group A and B/A variants bind EPCR (Turner et al., 2013). DC13 expressing parasites have been also found to present dual binding specificity for EPCR and ICAM1 (Lennartz et al., 2017). ICAM1 binding in DC13 has been mapped to the first DBLβ domain following the PfEMP1 head structure (Avril et al., 2016)(Figure 1.5). Whereas CD36 binding is restricted to group B and C head structures containing CIDRα2 to CIDRα6 domains (Hsieh et al., 2016; Turner et al., 2013; Yipp et al., 2007). Previous evidence also revealed that the DBLβ12 domain of 3D7_PDF0020c exhibiting PfEMP1 DC8 variant interacts with C1QBP (Magallon-Tejada et al., 2016). A computational modelling analysis of the DBLβ12 domain has predicted that the C1QBP binding site differs from the ICAM1 binding site found in other DBLβ subclasses (Bakri et al., 2021). Additional binding partners for other DC8 and DC13 domains remain to be identified (Avril et al., 2013). However, CM research field's intense focus on EPCR has meant that other host receptors contributing to brain sequestration are currently receiving little attention.

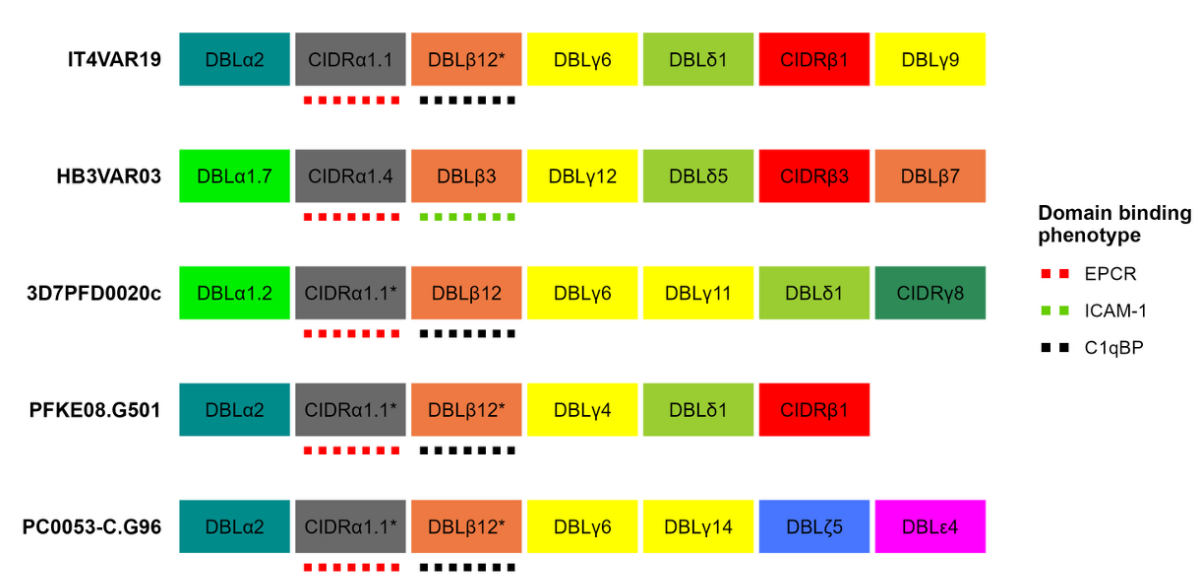


Figure 1-5. Illustration of some PfEMP1 HBEC binding domain architectures. IT4VAR19 is predicted to interact with C1QBP through its domain DBLβ12 (Magallon-Tejada et al., 2016). HB3VAR03 is also predicted to interact with ICAM1 (Janes et al., 2011; Lennartz et al., 2017). 3D7_PFD0020c, PfKE08, and PC0053-C. G96 (also known as 9197VAR27) are DC8-like PfEMP1 variants. The domain DBLβ12 of 3D7_PDF0020c is known

to bind C1QBP (Magallon-Tejada et al., 2016), while the PfEMP1 domains CIDRα1.1 and DBLβ12 with an asterisk are predicted to bind EPCR and C1QBP respectively. Computational analysis has predicted that DBLβ12 binding site on C1QBP (Black) differs to ICAM1 binding site (green) present in other DBLβ subclasses (Bakri et al., 2021). Some parasite lines expressing these PfEMP1 variants are studied in this thesis.

1.10.4. Host receptors involved in cytoadhesion

The identity of the major host endothelial receptor involved in CM remains controversial but may include EPCR (Turner et al., 2013) alone or in combination with ICAM1 (Avril et al., 2016; Berendt et al., 1989; Lennartz et al., 2017; Lennartz et al., 2019). Other endothelial cell receptors such as CD36 (Barnwell et al., 1985), P-selectin (CD62-P) (Udomsangpetch et al., 1997), Thrombospondin (TSP) (Roberts et al., 1985), PECAM1 (Platelet Endothelial Cell Adhesion Molecule 1) (Treutiger et al., 1997), E-selectin (CD62-E) and VCAM1 (Vascular Cell Adhesion Molecule 1) (Ockenhouse, Tegoshi, et al., 1992), Fractalkine (Hatabu et al., 2003), Integrin αvβ3 (Siano et al., 1998), CSA (Chondroitin Sulphate A) (Rogerson et al., 1995), NCAM (Neural Cell Adhesion Molecule) (Pouvelle et al., 2007) and C1QBP (Biswas et al., 2007) may also play a role in IE sequestration in diverse endothelial niches (Rowe et al., 2009). Host endothelial cell receptors, ECPR, ICAM1 and CD36 are the most extensively studied, while the others remain neglected. These three major receptors are described below, followed by the subject of this thesis, C1QBP.

1.10.4.1. EPCR

EPCR encoded by the endothelial protein C gene (*PROCR*) is a surface protein of human endothelial cells involved in anticoagulation and endothelial cytoprotective pathways through its interaction with protein C (PC) and activated protein C (APC) (Figure 1-3) (Mosnier et al., 2007). EPCR has been suggested as the major endothelial cell receptor for IE sequestration in the brain (Turner et al., 2013) and may act in conjunction with ICAM1 (Avril et al., 2016; Bernabeu & Smith, 2017; Lennartz et al., 2017; Lennartz et al., 2019). The N-terminal domain CIDRα1 of PfEMP1 has been identified as the binding partner of EPCR (Turner et al., 2013) and their interaction site predicted from X-ray crystallography structures using recombinant proteins (Lau et al., 2015). This interaction prevents the physiological function of EPCR in activation and maintaining endothelial barrier integrity, which induces coagulopathy, inflammation and vascular leakage contributing to severe malaria syndromes (Figure 1-3)

(Gillrie et al., 2015; Moxon et al., 2013; Sampath et al., 2015). Since the breakthrough discovery of Turner et al (Turner et al., 2013), the cerebral malaria research field has focused on EPCR, while other potential receptors are receiving little attention. Meanwhile, *in vitro* work has cast doubt on the physiological relevance of EPCR (Azasi et al., 2018) and the precise role of EPCR in adhesion to HBEC remains controversial, with results of adhesion assay differing between research groups (Azasi et al., 2018; Bernabeu & Smith, 2017; Gillrie et al., 2015; Storm et al., 2019; Turner et al., 2013). One possible explanation for these discrepancies has been demonstrated by the presence of complement C1s, a protease in the human serum that breaks PfEMP1 at interdomain conserved sites and inhibits cytoadherence of IE-EPCR (Azasi et al., 2021). It seems therefore possible that additional host receptors for IEs sequestration in the brain remain to be identified.

1.10.4.2. ICAM1

ICAM1 is a member of the immunoglobulin like superfamily, a 90-115 kDa glycosylated glycoprotein composed of an extracellular domain containing five tandemly arranged immunoglobulin-like domains, a transmembrane region, and a cytoplasmic domain (Staunton et al., 1988). ICAM1 surface expression can be stimulated both *in vitro* and *in vivo* on endothelial cells by TNF, interleukin-1 and gamma interferon (Avril et al., 2019). ICAM1 mediates the contact with leucocyte integrin, LFA-1 (Lymphocyte function-associated antigen 1) and Mac-1 (Macrophage-1 antigen) (Diamond et al., 1990) to facilitate the transmigration of lymphoid and myeloid blood cells through the vascular endothelium in response to tissue injury. ICAM1 is also a cellular receptor for the major human rhinoviruses (HRV) (Staunton et al., 1989), and a soluble form of ICAM1 binds to HRV and inhibits Rhinovirus infection (Marlin et al., 1990). Previous studies have demonstrated the ability of IEs to bind the first N-terminal domain of human ICAM1 (Berendt et al., 1992; Berendt et al., 1989; Ockenhouse, Betageri, et al., 1992). ICAM1 binding is mediated by a diverse set of DBLβ domains (Janes et al., 2011), which belong to PfEMP1 group A associated with severe malaria or group B and C linked to UM (Gullingsrud et al., 2013; Lennartz et al., 2017; Lennartz et al., 2019; Oleinikov et al., 2009). The cytoadhesion of IE to ICAM1 and the disease outcome remains a matter of controversy in the literature (Rowe et al., 2009). However, ICAM1 binding is associated with CM in some studies (Lennartz et al., 2017; Ochola et al., 2011) and more importantly it can act in combination with EPCR in *in vitro* and *ex vivo* studies (Avril et al., 2016; Lennartz et al., 2017; Lennartz et al., 2019; Tuikue Ndam et al., 2017). Specific monoclonal antibodies against

ICAM1 domain 1 and 2 competitively reduce cytoadherence of IEs (Davis et al., 2013; Madkhali et al., 2014). A more recent investigation used a well characterised parasite line associated with rosetting (PfKE08), expressing a DC8-like PfEMP1, and binding to ICAM1 in static adhesion assay (McLean et al., 2025). In general, ICAM1 binding is associated with severe malaria (Newbold et al., 1997).

1.10.4.3. CD36

CD36 is a scavenger receptor found on various host cells such as endothelium, monocytes, and platelets (Barnwell et al., 1989), but expressed at very low level or absent in the HBECs (Avril et al., 2013). This receptor has diverse functions including platelet adhesion (Biswas et al., 2007; Pain et al., 2001), participation in the fatty acid metabolic pathways, cell membrane trafficking systems and human innate immunity (Febbraio et al., 2001). Adhesion of IE to CD36 is mediated by CIDRα2-6 domains which belong to PfEMP1 group B, C and are associated with UM (Hsieh et al., 2016; Yipp et al., 2007). IE binding to CD36 is a common host-parasite cytoadhesion interaction, and 85% of PfEMP1 variants contain a CIDRα2-6 CD36-binding domain (Hsieh et al., 2016). Pediatric IEs isolates from UM cases have significantly higher levels of adhesion to CD36 compared to children with severe malaria cases (Ochola et al., 2011). In recombinant protein binding assays, IE selected on HBECs and predominantly expressing PfEMP1variants associated with severe malaria do not interact with soluble CD36 (Azasi et al., 2018). In general, CD36 binding is linked to UM (Newbold et al., 1997).

1.10.4.4. C1QBP

1.10.4.4.1. Structure of C1QBP

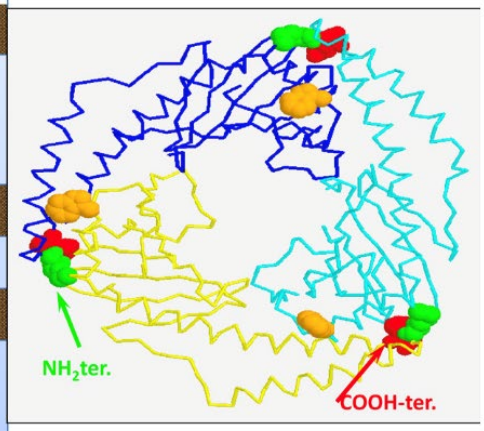
C1QBP also known as gC1qR (globular head domain of C1q receptor), HABP1 (Hyaluronan binding protein 1), p32 or p33 is encoded by a 6 kb gene named *C1QBP* and is composed of 6 exons and 5 introns localised at the short arm of chromosome 17 at position 17p13.3 (Guo et al., 1997). C1QBP protein migrates at 33 KDA on SDS-page (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) with a molecular weight of 97.2 KDA by gel filtration suggesting that the molecule is a trimer of three identical chain (Ghebrehiwet et al., 1994). Further, this finding was confirmed by the crystal structure of the mature form of C1QBP and

depicted to be a doughnut-shape homotrimer (Jiang et al., 1999) (Figure 1-6).

(A)

Other Names	**C1qBP,p33,p32, HABP**
Molecular weight	
SDS-PAGE Gel filtration Amino acids	33kDa 97.2kDa 24.3
Number of chains	**3 identical chains**
Isoelectric point	**4.15**
Number of amino acids	**282**
Gene structure Exons Introns	 6 5
Gene name	**C1qBP**
Chromosome location	**17p13.3**
Crystal structure	**Doughnut shaped homotrimer**

(B)



gC1qR trimer: Peptide backbones

(Jiang et al. PNAS, 1999).

Figure 1-6: Molecular and biochemical feature of C1QBP. A-) Description of the molecular and biochemical properties of C1QBP. **B-)** Shows the peptide backbones as well as the contacts between N- and C-termini in each monomer, which are critical for trimer formation. The figure is taken directly from (Ghebrehiwet et al., 2019).

The C1QBP protein is highly conserved in diverse species and shown 89.9% similarity of amino acid sequence between human and rodent (Guo et al., 1997). C1QBP contains 282 amino acid residues and contains a mitochondrial-targeting motif, the NH2 terminus of the mature protein isolated from Raji cell membranes starts at residue 74. Therefore, the hypothesis that the mature cell surface protein, spanning residues 74- 282 is a cleavage product of the pre-pro mitochondrial protein (i.e, the full length produced from mRNA translation containing both a signal peptide (pre) and an inactive sequence (pro)) residues 1-282 amino acid residues, which is translocated to the cell surface after post-translational modification without the first 74 amino acid residues (Ghebrehiwet et al., 2019; Ghebrehiwet et al., 1994).

1.10.4.4.2. C1QBP localisation and function

C1QBP is the physiological receptor for C1q, an important component of the complement system. The presence of receptor for C1q was first suggested on leucocytes in 1972 (Dickler & Kunkel, 1972) and demonstrated later with gC1q (globular head of C1q) binding with high affinity to C1QBP on diploid fibroblasts (Bordin et al., 1983). C1QBP is expressed on diverse human cells such as B cells (Ghebrehiwet et al., 1994), monocytes (Tenner & Cooper, 1981), macrophages (Malhotra & Sim, 1989), neutrophils (Eggleton et al., 1995), platelets (Peerschke & Ghebrehiwet, 1987), and endothelial cells (Andrews et al., 1981; Daha et al., 1988; Ghebrehiwet et al., 1994). However, the precise cellular localisation of C1QBP remains disputed in the literature. Multiple C1QBP cellular localisations may explain its broad assigned function (Figure 1-7).

- **Intracellular localisation of C1QBP and function**

Previous works found that the fluorescence of resting HUVEC cells labelled for C1QBP detection was minor but when HUVEC were permeabilised before staining more bright fluorescence was detected by flow cytometry. However, fixed HUVEC cells confirmed the predominant intracellular localisation of C1QBP using IFA (Dedio & Muller-Esterl, 1996; van den Berg et al., 1997).

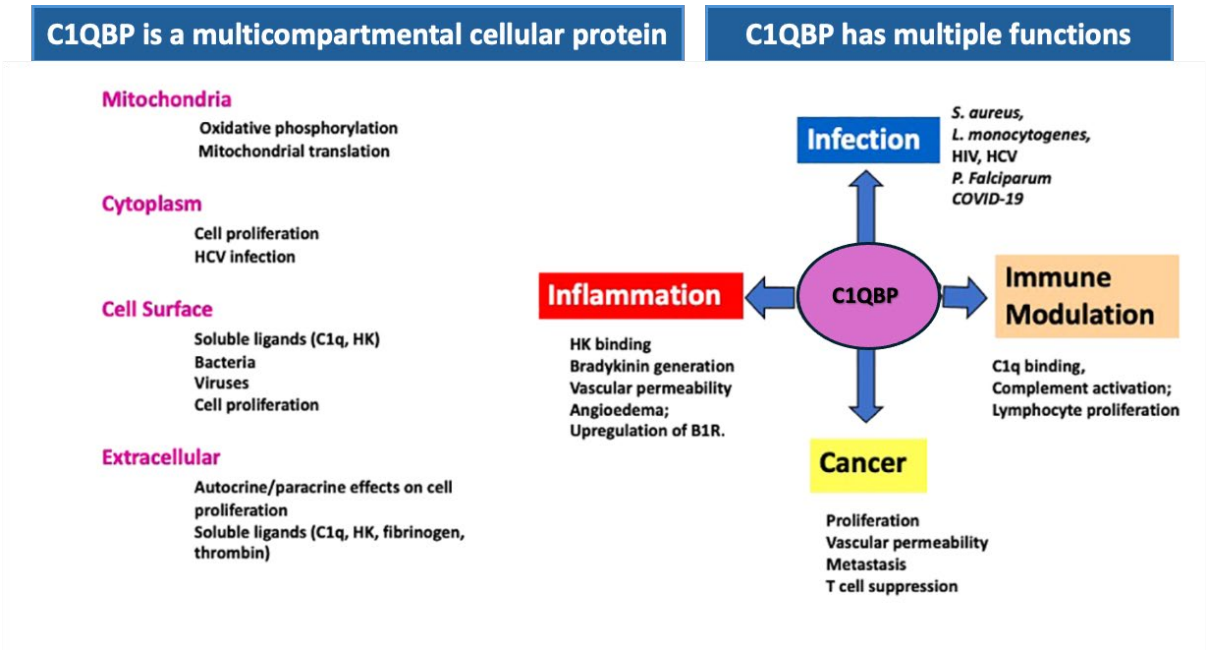


Figure 1-7: C1QBP multiple localisations and functions. Role of C1QBP in infection, inflammation, immune modulation, cancer, and diverse locations in cell compartment such as mitochondria, cytoplasm, cell surface and extracelluar environment are shown. C1QBP can interact with pathogens such as bacteria (Staphylococcus aureus, Listeria monocytogenes), virus (human immunodeficiency virus (VIH), hepatitis C virus (HCV), covid-19, and parasite (*P.falciparum*). C1QBP is a receptor for HK and induces the activation of the kinin kallikrein system (KKS) leading to the release of bradykinin, a potent vasoactive molecule known to cause angioedema. C1QBP in extracellular milieu can upregulated the expression of the inducible bradykinin receptor 1 (B1R). The figure is taken from (Ghebrehiwet et al., 2024).

The main intracellular site of C1QBP is in the mitochondrial matrix where it involves in oxidative phosphorylation (Muta et al., 1997). C1QBP facilitates dendritic cell metabolism and maturation, and acts in combination with pyruvate dehydrogenase to ensure the maturation of dendritic cells *in vitro* and *in vivo* (Gotoh et al., 2018). Development of malignant cells require substantial energy, knockdown of C1QBP has been shown to shift tumour cells metabolism from oxidative phosphorylation to glycolysis indicating that tumour cells use C1QBP to regulate the balance between oxidative phosphorylation and glycolysis (Fogal et al., 2010).

Another example of intracellular function of C1QBP is the protein ability to interact with Epstein-Barr virus (EBV) antigen called EBNA-1. This interaction allows the latent virus to use the host cellular machinery to transcribe his genetic material and facilitates viral infection (Wang et al., 1997). Intracellular C1QBP is known to interact with other viral protein to apply the same process such as HIV-1 Tat (human immunodeficiency virus) (Yu et al., 1995), HIV-1 Rev (Luo et al., 1994), and Herpes simplex virus (Bruni & Roizman, 1996).

- **Surface localisation and function**

Numerous studies have depicted C1QBP as a surface receptor (Andrews et al., 1981; Eggleton et al., 1995; Ghebrehiwet et al., 1994). However, in contrary to the classical cell surface receptors, C1QBP is missing a consensus motif for a transmembrane domain and a GPI (Glycosylphosphatidylinositol) anchor (Ghebrehiwet et al., 1994). Therefore, it is possible to attach to the cell surface from soluble C1QBP (Ghebrehiwet et al., 2014).

C1QBP has been shown to interact with multiple plasma protein like high molecular weight Kininogen (HK), Hageman factor (FXII) (Dedio & Muller-Esterl, 1996; Herwald et al., 1996; Joseph et al., 1996), multimeric vitronectin (Lim et al., 1996), fibrinogen and thrombin (Lu et

al., 1999) and contributes to both the intrinsic and extrinsic coagulation pathways. The significance of C1QBP as a cell surface mediator is targeted by bacterial and parasitic antigens to interact/ invade host cells (Ghebrehiwet et al., 2019).

C1QBP was first identified as a potential receptor for IEs by Biswas et al in 2007. They found that C1QBP is exhibited on the surface of endothelial cell using flow cytometry (Biswas et al., 2007). They have also demonstrated that clinical isolates and laboratory adapted parasite line (3D7_PFD0020c) selected on soluble C1QBP showed higher binding to recombinant C1QBP and HBECs than did non-selected clinical isolates and 3D7_PFD0020c parasites in spot and static HBEC adhesion assays. They used the selected IE (clinical isolates and 3D7_PFD0020c) in a HBEC-binding inhibition assay. Biwas et al found that monoclonal antibody to human C1QBP and recombinant C1QBP significantly reduced in a dose dependent manner the interaction between IEs and HBEC (Biswas et al., 2007). These results support the role of C1QBP as a potential receptor for IE sequestration in CM.

A subsequent study found that clinical isolates showing IE adhesion to C1QBP are associated with severe malaria presentation such as SMAn and multiple seizures, but the association with CM was not investigated due to insufficient number of samples (Mayor et al., 2011). Additional studies using laboratory adapted parasite lines showed that the selection of IT4VAR19-IE on immortalised HBEC (cell line HBEC-5i) led to the upregulation of the DC8-PfEMP1 variant IT4VAR19 and an increase in C1QBP binding in static adhesion assays (Claessens et al., 2012). Azasi et al also showed that IT4VAR19-IE binding to C1QBP in static adhesion assays is significantly increased in presence of 10% normal human serum (Azasi et al., 2018).

A complementary study also found that IE field isolates from Mozambican children experiencing severe malaria had a high transcript level of DC8 PfEMP1 and showed higher binding to recombinant C1QBP than children with UM (Magallon-Tejada et al., 2016). They also identified the DBLβ12 domain of the 3D7_ PDF0020c as the main ligand that mediates IE adhesion to C1QBP (Magallon-Tejada et al., 2016). Further computational analysis has identified specific amino acids within DBLβ12 domain of 3D7_PDF0020c that are predicted to bind to C1QBP, but this has not yet been confirmed by experimental data (Bakri et al., 2021).

Detailed study on the role of C1QBP in cytoadhesion of IEs selected for HBEC binding is lacking and more data on laboratory and clinical isolates are needed to support previous findings. This thesis addresses the role of C1QBP in IE cytoadhesion to HBEC.

1.10.5. Current strategies to investigate *P. falciparum* cytoadhesion

The use of new approaches to dissect the pathophysiology of CM have improved our understanding of the molecular interactions underlying the disease (Avril et al., 2012; Bernabeu et al., 2019; Claessens et al., 2012; Turner et al., 2013). *In vitro* models of cytoadherence of IE have been studied using target cells such as human umbilical vein endothelial cells (HUVECs) (Udeinya et al., 1981), C32 amelanotic melanoma cells (Schmidt et al., 1982), Chinese hamster ovary (CHO) cells (Oquendo et al., 1989), COS cells (Udeinya, 1990) transfected with genes coding for potential target receptors, and human dermal microvascular endothelial cells (HDMECs) (Siano et al., 1998). These cells are not brain endothelial cells but shared the ability to bind IE. Immortalised HBECs (Dorovini-Zis et al., 1991; Smith et al., 1992; Weksler et al., 2005) and primary HBECs have also been used (Avril et al., 2013; Avril et al., 2012; Biswas et al., 2007; Claessens et al., 2012; Claessens & Rowe, 2012).

Brain endothelial cells constitute the interface between intravascular and extravascular compartments and act as a selective barrier for the diffusion of cells and macromolecules between these two compartments and form with other cells the brain blood barrier (BBB) (Weksler et al., 2005). The endothelial cells that line the microvasculature of the brain share some features with endothelial cells from other organs, such as their capacity to modulate adhesion molecules in response to proinflammatory cytokines (Wassmer et al., 2006; Zougbede et al., 2011). However, HBECs also differ constitutively to those from other organs in the expression of tight junction molecules including claudin, occludins and ZO-1 (Wolburg & Lippoldt, 2002). Therefore, it is preferable to use human brain-derived endothelial cells to investigate IE cytoadherence to better characterise the ligand-receptor interaction that may occur in the brain during CM (Avril et al., 2012; Claessens et al., 2012).

Isolation of primary endothelial cells from human brain tissue is laborious, time-consuming, easily contaminated by fibroblasts and it is difficult to obtain sufficient cells with adequate confluency and viability (Navone et al., 2013). Consequently, immortalised HBECs such as HBEC-5i (Dorovini-Zis et al., 2011) and hCMEC/D3 (Weksler et al., 2005) were generated

and used as appropriate models for studying the biology of human brain endothelium in the context of neuroinflammatory or infectious diseases receptors based on cytoadherence, and for large-scale screening of CNS (Central Nervous System) drug candidates (Weksler et al., 2013; Weksler et al., 2005). They offer several advantages including continuous culture through at least 35 passages, and the ability to cryopreserve viable cells at different passages for later thawing and continuous culture (Weksler et al., 2005). Cells from all passages grow well and reach confluency in 2 to 3 days after thawing or splitting, with no contamination by fibroblasts, and they retained the normal phenotypic characteristic of HBEC such as exhibiting intracellular positive staining to vWF and negative staining to SMA (Navone et al., 2013; Weksler et al., 2005). However, the transduction of foreign genes (human telomerase and SV40 large T antigen) required for immortalisation may affect the expression of antigenic determinants, cell surface molecules, metabolic properties, and permeability functions (Navone et al., 2013).

Researchers have used a variety of different cell lines to identify the endothelial receptors involved in IE cytoadherence. In all cases it is essential to characterise the endothelial nature of the primary or immortalised HBECs prior to investigate IE cytoadhesion. Numerous studies have validated the endothelial nature of HBECs by staining cells with specific endothelial cell markers such as vWF, PECAM1/CD31, Dil-Ac-LDL, and SMA before further investigations (Avril et al., 2013; Azasi et al., 2018; Navone et al., 2013; Wassmer et al., 2006; Weksler et al., 2005).

The detection of surface expression of putative cytoadhesion receptors on HBECs was assessed after the confirmation of HBECs endothelial origin. Researchers have performed immunofluorescence assay (IFA)/ flow cytometry/ ELISA (Enzyme linked Immunosorbent Assay) (Avril et al., 2012; Biswas et al., 2007; Claessens et al., 2012; Wassmer et al., 2011; Xiao et al., 1996) to localise candidate receptors. Activated HBECs stimulated with proinflammatory cytokines such as TNF-α were compared with resting HBECs to evaluate the change in expression of specific receptors (Navone et al., 2013; Weksler et al., 2005; Xiao et al., 1996; Zougbede et al., 2011). In general, ICAM1 was upregulated and EPCR dowregulated after HBECs activation with TNF-α (Simmonds & Lane, 1999; Wassmer et al., 2006; Zougbede et al., 2011)

Binding of laboratory culture-adapted parasite lines and field isolates on HBECs has been investigated with cytoadherence assays under static conditions and using flow-based experiments that attempt to reproduce constant shear forces like those experienced *in vivo* (Azasi et al., 2018; Claessens & Rowe, 2012; Cojean et al., 2008; Mahamar et al., 2017; Ponsford et al., 2012; Siano et al., 1998; Udeinya et al., 1981). The development and use of three-dimensional (3D) cell culture system have offered the possibility to investigate the molecular mechanisms of IEs cytoadhesion using engineered 3D microvessels (Bernabeu et al., 2019). In this model, 3D microvessels are seeded with primary HBEC in a microfluidic network and perfused to generate a wide range of physiologically relevant blood flow shear forces. When the HBECs are confluent, the device is perfused with IEs, EPCR and ICAM1-mediated binding of culture-adapted parasite lines have been demonstrated (Bernabeu et al., 2019). The combination of endothelial cells, pericytes and or astrocytes into a co-culture model has been used to investigate IE cytoadhesion for a better characterisation of host-parasite interaction during CM (Storm et al., 2020). Other group used a 3D spheroid BBB model to show that IE exhibiting dual binding to EPCR and ICAM1 can cross the BBB (Adams et al., 2021).

Cytoadhesion assays have also been widely used in a panning strategy (to enrich *P. falciparum* strains for adhesion to HBEC) with multiple rounds of selection (Avril et al., 2012; Claessens et al., 2012). Selected parasite lines can be used for the molecular characterisation of host-parasite adhesion interactions (Claessens et al., 2012). Cytoadhesion inhibition assays can also be carried out, in which specific antibodies to candidate host receptors are added to HBEC cultures before the addition of IEs and compared with a control without the addition of specific antibodies, to estimate the specific blocking effect on cytoadhesion (Avril et al., 2016; Avril et al., 2013; Avril et al., 2012; Azasi et al., 2018; Biswas et al., 2007; Claessens et al., 2012; Turner et al., 2013; Xiao et al., 1996). In addition, for inhibition of specific putative surface receptors, HBECs can be transfected with a siRNA (silencing RNA) or an irrelevant siRNA (scramble siRNA) to reduce expression of the receptor on the surface of HBECs, followed by cytoadhesion assay and assessment of binding intensity (Azasi et al., 2018). The goal of such studies is to identify endothelial cell receptors involved in IE cytoadhesion mechanisms, and examples of some of these assays will be given later in the thesis.

1.11. Rationale for studying the role of C1QBP in adhesion to HBEC

This background chapter highlights a particular concern about the role of EPCR as the only major host endothelial receptor for IE sequestration in CM. IE binding to EPCR via the CIDRα1 domain of some PfEMP1 has been associated with severe and CM in children (Lavstsen et al., 2012; Turner et al., 2013). However, a major study found that in the presence of normal human serum, the interaction between IE and EPCR is disrupted. This finding cast doubt on the physiological relevance of EPCR as the main endothelial receptor for IEs during CM (Azasi et al., 2018). Therefore, this research looks for another potential host receptor. Previous studies have identified C1QBP as a potential candidate that can promote IE adhesion to HBEC (Biswas et al., 2007; Magallon-Tejada et al., 2016; Mayor et al., 2011). However, there are very few studies on C1QBP and malaria in the literature, and it remains unknown whether IE selected on HBEC adhesion to C1QBP plays a role in CM. This thesis uses the immortalised HBEC (hCMEC/D3) (Weksler et al., 2005) to investigate the role of host C1QBP in IE sequestration.

1.12. Hypothesis and aims

The hypothesis to be investigated is that C1QBP is an endothelial receptor for *P. falciparum* IE cytoadhesion to HBEC. This will be tested *in vitro* using laboratory culture-adapted parasite lines and immortalised HBEC. The aims of the study are:

1. To determine whether C1QBP is expressed by HBECs.
2. To determine whether HBEC-selected *P. falciparum* IEs expressing the "domain cassette 8" type of PfEMP1 use C1QBP as a cytoadhesion receptor.

2. Chapter II: Materials and Methods

This chapter describes the materials and general methods for parasite and cell culturing, binding assays, immunofluorescent staining of HBECs and IEs, selection of parasites for binding to HBECs and HBEC inhibition assays. I also describe an ELISA for soluble C1QBP detection and quantification in normal human plasma. The methods for data visualisation and graphing and statistical approaches used for data analysis are also described.

2.1. Ethical permissions

The pooled human serum and blood used in this study were obtained from the Scottish National Blood Transfusion Service (SNBTS), Edinburgh, UK, with ethical permission reference number 19~08 and 22~11.

2.2. Immortalised and primary human endothelial cell lines

To examine IE cytoadherence, I used the immortalised HBEC line hCMEC/D3 (Sigma, SCC066) as a model for understanding the parasite adhesin-host receptor interactions that may occur in the human brain microvasculature during IE infection. hCMEC/D3 is an immortalised microvascular HBEC line isolated from temporal lobectomy procedures on an adult female with epilepsy (Weksler et al., 2005). To become immortalised, these endothelial cells were transduced by lentiviral vectors incorporating human telomerase or SV40T antigen. After sequential dilution cloning of the transduced cells, one was selected for expansion due to expression of normal human endothelial markers, such as CD31, VE cadherin, and vWF, the most specific marker for endothelial cells. This endothelial cell line grew normally after 35 passages without any phenotypic dedifferentiation (Weksler et al., 2005), and is used throughout this thesis. Other human endothelial cell lines such as the immortalised HBEC line, HBEC-5i (Dorovini-Zis et al., 1991), primary lung microvascular endothelial cell (HPMEC), primary HDMEC, and primary brain microvascular endothelial cell (HBMEC) were also used to characterise the cellular localisation of C1QBP. All work with HBECs was performed in a class II biological safety cabinet under sterile conditions. All materials used for culturing were sterile and solutions were warmed to 37°C before use.

2.2.1. hCMEC/D3 culturing

hCMEC/D3 cells were grown in 1% collagen (Merck Millipore, 08-115)-coated vented tissue culture flasks (Corning®, 430168). The flasks were coated with collagen by adding 3 mL of

1% (v/v) collagen in PBS (Phosphate Buffer Saline) (Sigma, P4417) into a 25 cm^2 vented flask and incubating the flask at 37°C for at least 30 minutes. After incubation, the collagen solution was removed by aspiration. Incomplete DMEM (Dulbecco's Modified Eagle's Medium)/F-12 Ham medium (Sigma, D6421) containing 15 mM Hepes and 15 mM sodium bicarbonate was used, and was supplemented with 2 mM L-glutamine (Gibco, 25030) and 1% penicillin/streptomycin 100x (ScienCell, 0503). To obtain complete DMEM/F-12 medium, incomplete medium was supplemented with 10% (v/v) heat-inactivated fetal bovine serum (ScienCell, 0025) and 1% (v/v) endothelial cell growth supplement (ScienCell, 1052). Cells were grown at 37ºC under 5% CO_2 in air, and the medium was replaced every two days. The cells were lifted at a confluency between 80 to 100% as assessed using an inverted microscope at 20X (Olympus, Ck2).

Reagents used to grow HBEC-5i were the same as for hCMEC/D3 culture. However, for primary human endothelial cells, endothelial cell medium (ECM) (ScienCell, 1001) instead of DMEM/ F-12 Ham with additives as above was used, as recommended by the supplier.

2.2.2. hCMEC/D3 lifting

The confluent hCMEC/D3 cells (80-100%) were detached after twice washing with incomplete DMEM medium. The washing solution was swirled over the cells and removed by aspiration. Two mL of 0.25% trypsin-EDTA solution (ScienCell, 0103) for a 25 cm^2 flask was added and incubated for 3 minutes at 37°C. After incubation, the flask bottom was hit to detach adherent cells, and 10 mL of complete medium was added to inhibit the trypsin activity. The mixture was transferred into a Falcon 15 mL tube for centrifugation at 650 x g for 4 minutes. If done properly, the bottom of the flask should be clear when viewed under an inverted microscope. The pellet of cells was resuspended thoroughly in 10 mL complete medium and returned to culture after a further 1:5 dilution in complete medium (10 mL into a 25 cm^2 flask or 25 mL into a 75 cm^2 flask).

The same methods described above were performed for other endothelial cell lines, immortalised HBEC-5i and primary endothelial cells (HBMEC, HPMEC, and HDMEC).

2.2.3. hCMEC/D3 freezing

To cryopreserve hCMEC/D3 cells, confluent hCMEC/D3 cells were trypsinised as described above. After washing once with 10 mL of complete medium, hCMEC/D3 cells were

resuspended in cell freezing medium (Sigma, C6164) to obtain around 1 x 10^6 cell /mL. This was determined by placing 10 µL of resuspended cells in complete medium on a haemocytometer (Neubauer improved, BS.748 Blaubrand). Cells were counted in 2 large squares and determined the average number of cells denoted as Z. The number of cells was calculated using the following formula, the number of cells = Z x 10^4 cell/ mL. Cells were diluted in cell freezing medium, mixed well and frozen approximately at 1 x 10^6 cell/ mL into a Nunc cryogenic vial at -70°C for a short-term, before being transferred to liquid nitrogen for long-term storage.

The same cell freezing procedures were applied to other endothelial cells studied.

2.2.4. hCMEC/D3 thawing

A vial of frozen hCMEC/D3 was thawed in a 37°C water bath, then the cells were resuspended in 10 mL complete medium and centrifuged at 650 x g for 4 minutes. The supernatant was removed, and cells were resuspended in 10 mL complete medium placed in a small flask (25 cm^2) with a vented lid in a 5% CO_2 incubator. It is important to resuspend the cells by pipetting up and down to avoid the cells forming clumps on the bottom of the flask instead of being properly distributed.

The same cell thawing methods were carried out for immortalised (HBEC-5i) and primary endothelial cells (HBMEC, HPMEC, and HDMEC).

2.3. hCMEC/D3 cell surface characterisation using immunofluorescence assay (IFA)

To stain for receptors on the surface of hCMEC/D3 cells, I performed an immunofluorescence assay (IFA). IFA involves the combination of labelling proteins using a first specific antibody, followed by a secondary antibody specific to the isotype of the first antibody and linked to a fluorescent dye.

hCMEC/D3 cells suspension (500 µL/ per well) were seeded at 2.5-5 x 10^4 cell/ mL in 8 well chamber slides (Thermo Fisher Scientific, 15494) precoated with 1% rat tail collagen I (Merck Millipore, 08-115) and grown to 80-100% confluence for 2-3 days. After 24-48 hours, the chamber slide medium was discarded and cells were washed once with 500 µL of PBS (Sigma, P4417). Half of the wells were stimulated with TNF-α (R&D systems, 210-TA) at 100 ng/mL for 24 hours in cell culture medium and the remaining half wells contained only cell culture

medium. After 24 hours incubation, cells were washed once with 500 µL of PBS and fixed with PBS/1% paraformaldehyde (Thermo Fisher Scientific, 28908) for 15 minutes at RT (Room Temperature) and washed once again with 500 µL of PBS. Cells were blocked with 500 µL of 5% goat serum (Capricorn Scientific, GOA-1B) for 30 minutes at RT, then washed once with 500 µL of PBS. Cells were incubated for 1 hour at RT with 200 µL of PBS/1% BSA (Bovine serum albumin) (Sigma, A8577) containing specific primary antibody at a concentration recommended by the manufacturer (Table 2-1). Stained cells were washed three times with 500 µL of PBS and incubated with 200 µL of PBS/1% BSA containing an appropriate secondary antibody at 1:500 final concentration (Alexa Fluor 488 goat anti-mouse IgG (Thermo fisher Scientific, A11029) or Alexa Fluor 488 goat anti-rat IgG (Molecular probes, A11006) or Alexa Fluor 488 goat anti-rabbit IgG (Invitrogen, A11034) or Alexa Fluor 488 goat anti-human IgM (Invitrogen, A21215); and 1 µg/mL DAPI (4',6 diamidino-2-phenyllindole) (Molekula, M70277046) for 45 minutes in the dark. Cells were washed three times with 500 µL of PBS and well chambers removed. A drop of fluoromount (Sigma, F4680) was added to each well and the slide was covered with a 22 x 50 mm coverslip (Scientific Laboratory Supplies, MIC3226).

The slide was left to set at 4°C in the dark until the next day. Omission of the primary antibody and an appropriate irrelevant IgG served as negative controls in these experiments. The slides were viewed with a fluorescent microscope (Leica, DM LB 2 or Zeiss, Axioskop 2 plus) using the 40X or 100X objective. Images were acquired with a DFC300FX digital camera (Leica, DM LB 2) or Retiga 2000R (Zeiss, Axioskop 2 plus) using the same exposure time and camera setting for all samples and negative controls. Images were saved in TIFF format as unprocessed pictures and were imported into image J (**https://imagej.net/ij/**) to perform images analysis as described below.

- Image ⇒ adjust ⇒ brightness/ contrast
- Image ⇒ color ⇒ merge channels
- Analyze ⇒ tool ⇒ scale bar (20 µm)

All setting adjustments were applied identically to stained wells and negative controls. Analysed images were saved as processed pictures in TIFF format. The presence or absence of fluorescent signal was determined visually and given a score from ‹‹-» (no signal) to ‹‹+» positive signal. Staining with specific antibody was compared to that of the two negative controls.

To test the ability of soluble C1QBP (Sino Biological, 11874) to become attached to the surface membrane of hCMEC/D3 and other endothelial cells, HBEC cells were preincubated with soluble C1QBP at 2.5 μg/mL in cell culture medium for 2 hours before IFA as described above. To test also the biological relevance of this finding, hCMEC/D3 cells were preincubated with 10% normal human plasma in complete DMEM/F-12 medium for 24 hours before IFA as described above.

Table 2-1. Antibodies to endothelial cell markers and candidate cytoadhesion receptors used in IFAs

Antigen	**Clone**	**Supplier/ order number**	**Isotype**	**Clonality**	**Initial concentration**	**Manufacturer concentration for IFA**
Control mouse IgG1	MOPC-21	Abcam/ 18443	Mouse IgG1	Monoclonal	0.5 mg/mL	20 μg/mL
ICAM1 (CD54)	15.2	Santa Cruz Biotechnology/ 107	Mouse IgG1	Monoclonal	1 mg/mL	20 μg/mL
CD36	FA6- 152	Hycult Biotechnology/ HM2122	Mouse IgG1	Monoclonal	0.1 mg/mL	20 μg/mL
Smooth Muscle Actin (SMA)	B4	Santa Cruz Biotechnology/ 53142	Mouse IgG1	Monoclonal	0.2 mg/mL	20 μg/mL
PECAM1 (CD31)	WM59	Biolegend/ 303102	Mouse IgG1	Monoclonal	1 mg/mL	10 μg/mL
VCAM1 (CD106)	STA	Biolegend/ 305802	Mouse IgG1	Monoclonal	0.5 mg/mL	20 μg/mL
C1QBP	MM09	Sino Biological/ 11874	Mouse IgG1	Monoclonal	1 mg/mL	25 μg/mL
E-selectin (CD62E)	P2H3	Abcam/ 24723	Mouse IgG1	Monoclonal	1 mg/mL	10 μg/mL
E-selectin (CD62P)	CLB-Thromb/6	Immunotech/ IM1315	Mouse IgG1	Monoclonal	0.5 mg/mL	10 μg/mL
Integrin αv (CD51)	AMF7	Immunotech/ IM0770	Mouse IgG1	Monoclonal	0.2 mg/mL	20 μg/mL
Integrin αv (CD51)	468255	Abcam/ 55991	Rabbit IgG	Polyclonal	0.5 mg/mL	20 μg/mL

Fractalkine CX3CL1	AXV02	R&D systems/ AF365	Goat IgG	Polyclonal	0.1 mg/mL	10 µg/mL
Thrombospondin (TSP)	A6.1	Invitrogen/ 399300	Mouse IgG1	Monoclonal	0.5 mg/mL	20 µg/mL
NCAM (CD56)	MEM-188	Biolegend/ 304604	Mouse IgG2a, k	Monoclonal		5µL per100 µL staining volume
CSA	CS56	Sigma/ C8035	Mouse IgM	Monoclonal	1.7 mg/mL	5 µg/mL
Control rabbit IgG		R&D systems/ AB-108-C	Rabbit IgG	Polyclonal	1 mg/mL	10 µg/mL
VWF		DAKO/ A0082	Rabbit IgG	Polyclonal	1 mg/mL	10 µg/mL
C1QBP	RP01	Abcam/ 101267	Rabbit IgG	Polyclonal	1 mg/mL	5 µg/mL
Control IgG rat	RTK2071	Biolegend/ 400402	Rat IgG1	Monoclonal	0.5 mg/mL	20 µg/mL
α EPCR (CD201)	RCR- 252	BD pharmingen/ 552500	Rat IgG1	Monoclonal	0.5 mg/mL	20 µg/mL
α EPCR	RCR- 252	Hycult biotech/ HM2145	Rat IgG1	Monoclonal	0.1 mg/mL	20 µg/mL
Control mouse IgG2a, K	M0PC-173	Biolegend/ 400202	Mouse IgG2a, K	Monoclonal	0.5 mg/mL	20 µg/mL
Dil- Ac-LDL		Life technology/ L3484			1 mg/mL	15 µg/mL

C1QBP: Complement C1q binding protein; **CSA:** Chondroitin sulfate A; **Dil-Ac-LDL:** Dil acetylated low density lipoprotein; **EPCR:** Endothelial protein C receptor; **ICAM1:** Intercellular adhesion molecule 1; **NCAM:** Neural cell adhesion molecule; **PECAM1:** Platelet endothelial cell adhesion molecule 1; **SMA:** Smooth muscle actin; **TSP:** Thrombospondin; **VCAM1:** Vascular cell adhesion molecule 1; **vWF:** von Willebrand factor.

2.4. Sandwich enzyme linked immunosorbent assay for C1QBP detection and quantification

To determine whether soluble C1QBP is present in normal human plasma, I used a commercial ELISA test kit (FineTest, Wuhan fine Biotech, EH1080).

2.4.1. Principle of the assay

ELISA kit test (FineTest, Wuhan fine Biotech, EH1080) used capture antibody pre-coated onto 96 well plates and biotin conjugated antibody was used as antibody detection. Standards, test samples, and biotin conjugated antibody detection were subsequently added to wells and

washed with washing buffer. HRP-streptavidin (horseradish peroxidase conjugated streptavidin) was added, and unbound conjugates were washed away with washing buffer. TMB (tetramethylbenzidine) substrates were used to visualize HRP enzymatic reaction. TMB was catalysed by HRP to produce a blue color product that changed into yellow after adding sulfuric acid (H_2SO_4) stop solution. The density of yellow color is proportional to the target amount of sample captured on the plate. Read OD (optical density) absorbance at 450 nm in a microplate reader, and then the concentration of targeted sample can be calculated.

Table 2-2: Kit components and storage

No.	**Item**	**Specifications** (96T)	**Storage**
E001	ELISA microplate (Dismountable)	8x12	2-8°C/ -20°C
E002	Lyophilised standard	2 vials	2-8°C/ -20°C
E039	Sample dilution buffer	20 mL	2-8°C
E003	Biotin-labeled antibody (Concentrated, 100X)	120 μL	2-8°C (avoid direct light)
E040	Antibody dilution buffer	10 mL	2-8°C
E034	HRP- streptavidin conjugate (SABC, 100X)	120 μL	2-8°C (avoid direct light)
E049	SABC dilution buffer	10 mL	2-8°C
E024	TMB substrate	10 mL	2-8°C (avoid direct light)
E026	Stop solution	10 mL	2-8°C
E038	Wash buffer (25X)	30 mL	2-8°C
E006	Plate sealer	5 pieces	

2.4.2. Samples collection, storage, and dilution

I collected whole blood with tube containing ACD as an anticoagulant (BD vacutainer, 366645). I centrifuged tube at 1000 x g at 2-8°C within 30 minutes of blood collection. I collected supernatants (plasma) and I conducted the assay immediately. Supernatants can also be aliquoted and stored at -20°C or -80°C for future assay.

2.4.3. Samples dilution

I selected a proper dilution factor to make the diluted C1QBP concentration fall in the optimal detection range of the kit (0.156-10 ng/mL). I diluted samples with the provided dilution buffer (E039). I mixed samples very well with the dilution buffer. I prepared standard curve and test samples before the experiment. If samples had very high concentration, I diluted samples with PBS first and secondly with dilution buffer.

I randomly selected the human plasma samples from those used in a recent study (McLean et al., 2024) and were collected as part of protocols approved by the University of Edinburgh, School of Biological Sciences Ethical Review Panel (arowe002) and Ghana Health Service Review Committee ID No. GHS-ERC: 03/05/14 and Noguchi Memorial Institute for Medical Research-IRB study# 020/13–14.

2.4.4. Reagents preparation and storage

I kept all reagents and samples at RT for 20 minutes before the assay.

- **Washing buffer (25X)**

I diluted concentrated washing buffer (25X) with distilled water to obtain (1X). for example, I diluted 30 mL concentrated washing buffer with distilled water to have 750 mL. I stored the rest of washing buffer solution at 2-8°C.

- **Standards**

I centrifuged a standard tube for 1 minute at 10000 x g, and labeled it as zero tube. I added standard dilution buffer (1 mL) into one lyophilised standards tube, I tightened the standards tube cap and let it stand for 2 minutes at RT. I inverted the tube several times to mix gently. I centrifuged the tube for 1 minute at 1000 x g, making the liquid towards the bottom of the tube and removing possible bubbles.

To dilute the standard, I labeled seven Eppendorf tubes as 1/2, 1/4, 1/8, 1/16, 1/32, 1/64, and blank respectively. I added 0. 3 mL of the sample dilution buffer into each tube. Then, I added 0.3 mL of the above standard dilution buffer labeled as zero tube into tube labeled 1/2 and mixed thoroughly. I transferred 0.3 mL from 1/2 tube into 1/4 tube and mixed vigorously. I

transferred 0.3 mL from 1/4 tube into 1/6 tube and mixed strongly, so on till 1/64 tube. Blank tube only contained 0.3 mL of sample dilution buffer. The standard concentration from zero tube to blank tube is 10 ng/mL, 5 ng/mL, 2.5 ng/mL, 1.25 ng/mL, 0.625 ng/mL, 0.312 ng/mL, 0.156 ng/mL, and 0 ng/mL (Figure 2-1).

I stored the zero tube with dissolved standard at 2-8°C and used within 12 hours. I used other diluted working solutions containing standard in 2 hours.

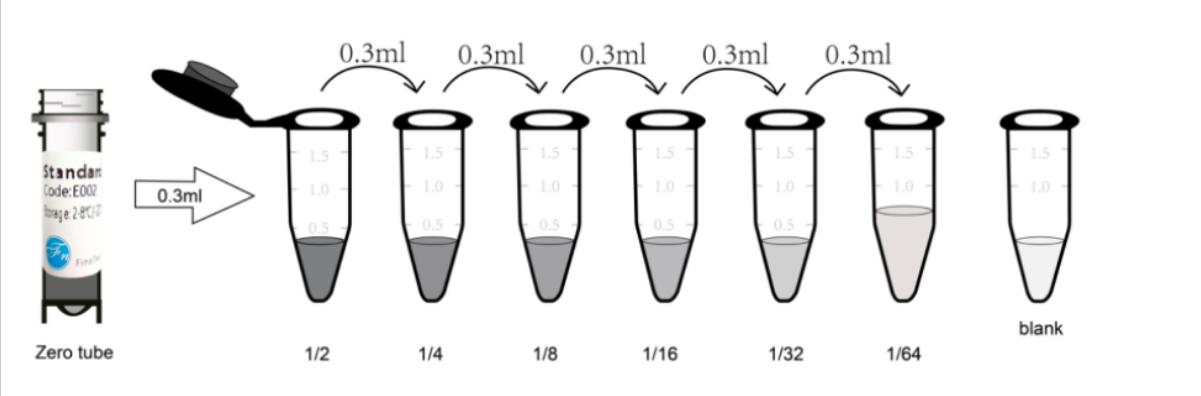


Figure 2-1: Preparation of standard dilution. The figure is taken directly from www.fn-test.com.

- **Preparation of biotin-labeled antibody working solution**

I prepared the working solution within 30 minutes before the assay and can't be stored after the assay. I calculated the total volume of the working required solution: 100 µL/well x quantity of wells. I centrifuged the working solution for 1 minute at 1000 x g in low speed and bring down the concentrated biotin-labeled antibody to the bottom of tube. I diluted the biotinylated detection antibody with antibody dilution buffer at 1:99 and mixed vigorously (e.g. Add 10 µL concentrated biotin-labeled antibody into 990 µL antibody dilution buffer.)

- **Preparation of HRP-streptavidin conjugate (SABC) working solution**

I prepared the working solution within 30 minutes before the assay and can't be stored after the assay. I calculated the total volume of working solution required: 100 µL/well x quantity of wells. I centrifuged the working solution for 1 minute at 1000 x g in low speed and bring down the concentrated SABC to the bottom of tube. I diluted the concentrated SABC with

SABC dilution buffer at 1:99 and mixed vigorously (e.g. Add 10 µL concentrated SABC into 990 µL SABC dilution buffer.).

2.4.5. Assay procedure

Firstly, I designed the assay plate (Table 2-3) and set standard, samples, and control (blank) wells on the pre-coated plate in duplicate. I aliquoted 100 µL of serial diluted standards (described above) in corresponding wells. I also added 100 µL sample dilution buffer into the control (blank) wells. Then, I prepared 100 µL plasma samples of unknown C1QBP concentration neat and at 1/2 and 1/4 dilution in sample dilution buffer as above. I sealed the plate and incubated for 90 minutes at 37°C (Table 2-3). After the incubation, I removed the plate cover, aspirated the liquid in the plate, and added 350 µL wash buffer into each well without immersion. I discarded the liquid in the well and tap on the absorbent paper. I repeated the washing step twice. After the washing step, I added 100 µL biotin-labeled detection antibody working solution in each well, sealed the plate, and incubated for 60 minutes at 37°C. After aspiration and washing three times as described above , I incubated wells with 100 µL of HRP-streptavidin conjugate (Secondary antibody conjugate) for 30 minutes at 37°C. Then, I washed wells five times as above, and I detected bound antibody by incubation for 15 minutes at 37°C with the substrate for HRP, 3,3',5,5'-Tetramethylbenzidine dihydrochloride hydrate (TMB). I stopped the reaction by adding 50 µL of stop solution (H_2S0_4) in each well and immediately, I read the OD (optical density) absorbance at 450 nm in a microplate reader (FluoStar Omega, BMG Labtech GmbH, Germany).

Table 2-3. Design ELISA plate assay

	1	2	3	4	5	6	7	8
A	Blank	Blank	Blank	Blank	AH012	1/4	AH032	1/4
B	0.156 ng/mL	0.156 ng/mL	1/4	1/4	AH012	1/2	AH032	1/2
C	0.312 ng/mL	0.312 ng/mL	1/2	1/2	AH011	1/4	AH030	1/4
D	0.625 ng/mL	0.625 ng/mL	1/4	1/4	AH011	1/2	AH030	1/2
E	1.25 ng/mL	1.25 ng/mL	1/2	1/2	AH009	1/4	AH027	1/4
F	2.5 ng/mL	2.5 ng/mL	1/4	1/4	AH009	1/2	AH027	1/2
G	5 ng/mL	5 ng/mL	1/2	1/2	AH006	1/4	AH025	1/4
H	10 ng/mL	10 ng/mL	Normal	**Normal**	AH006	1/2	AH025	1/2
	Standard		**Normal human plasma**		**Infected patients' plasma**		**Infected patients' plasma**	

: Normal human plasma collected freshly

: Normal human plasma collected and stored at 4ºC for three weeks

: Normal human plasma collected and stored at -70ºC for three weeks

AH000: Identification number of plasma collected from patient infected with *P. falciparum.*

Sample dilution factor 1/2

Sample dilution factor 1/4

2.4.1. ELISA analysis of soluble C1QBP concentration

To assess quantitatively soluble C1QBP in normal human and *P. falciparum* infected patients' plasma, I imported the raw data of OD absorbance at 450 nm generated by FluorStar microplate reader in an Excel format (Table 6-1, appendix). I calculated the average of OD absorbance for

each standard, sample, and blank, then the corrected OD absorbance for each standard and sample (e.g. corrected OD of a specific sample = average OD value of that sample – average value of the blank) (Table 6-1, appendix). I used GraphPad Prism version 9.5.1 to generate the standard curve and determine the concentration of unknown samples. I plotted the standard curve as the corrected OD absorbance 450 nm of each standard solution (y axis) versus C1QBP concentration (x axis). I extrapolated the concentration of each sample from the standard curve, if sample was diluted, I multiplied the concentration read from the standard curve by the dilution factor.

2.5. *P. falciparum* strains

Four culture-adapted parasite strains, IT4, HB3, PfKE08 (also known as 9605VAR2) and PC0053-C.G96 (also known as 9197VAR27) were used. IT4 was originally isolated from a malaria patient in Brazil in 1979 (Udeinya et al., 1983). However, the existing IT4 parasite line used globally for malaria research is thought to be a product of cross contamination with a fast-growing southeast Asian strain (Mu et al., 2005; Roberts et al., 1992; Robson et al., 1993; Trager, 1993). HB3 was isolated from a patient in Honduras in 1980 (Bhasin & Trager, 1984). IT4 and HB3 have been previously selected for binding to HBEC-5i (Claessens & Rowe, 2012), and respectively expressed the PfEMP1 variants IT4VAR19 (PFIT_010005000, DC8) and HB3VAR03 (PFHB3_130080100, DC13) (Azasi et al., 2018; Claessens et al., 2012) (Figure 1-5). PfKE08 and PC0053-C.G96 were isolated from malaria patients in Kenya (McLean et al., 2025). These parasite lines (PfKE08 and PC0053-C.G96) were used in this study for their ability to express the DC8 PfEMP1 variants illustrated in Figure 1-5.

2.5.1. *P. falciparum* culture

All work with *P. falciparum* was performed under biosafety level 3 with derogations. Culturing was carried out in a Class II biological safety cabinet under sterile conditions. All materials used for culturing were sterile and solutions were warmed up in a water bath at 37°C before use.

2.5.2. *P. falciparum* in vitro culturing

Parasite lines were cultured in group O^+ erythrocytes (SNBTS, Edinburgh, Scotland) at 2% haematocrit (Ht) in a complete RPMI (Roswell Park Memorial Institute) medium. Incomplete RPMI medium consisting of RPMI 1640 containing sodium bicarbonate (Lonza, BE12-167F),

supplemented with 16 mM glucose (Scientific Laboratory Supplies, CHE1806); 2 mM L-glutamine (Gibco, 2530-081); 25 mM Hepes (Lonza, BE17-737E); 25 μg/mL gentamycin sulfate (Lonza, 17-518Z); and adjusted to pH 7.2-7.4 with 1 M NaOH. To obtain complete RPMI medium, incomplete medium was supplemented with 0.25% Albumax II lipid-rich bovine serum albumin (BSA) (Thermo Fisher, 11021037); and 5% pooled normal human serum (SNBTS). The parasite cultures were maintained in sterile non-vented flasks of diverse sizes: 25 cm^2 (Corning®, 430168); 75 cm^2 (Corning®, 430720); and 150 cm^2 (Corning®, 430825) for maximum culture volume of 15 mL; 40 mL; and 100 mL respectively. The culture flasks were gassed with 1% O_2, 5% CO_2 and 96% N2 (BOC, 280648-L) and incubated at 37°C.

The culture medium was changed daily by centrifuging the culture suspension in a Falcon tube at 1000 x g for 5 minutes, and the supernatant aspirated. The parasite pellet was then resuspended in fresh complete medium to achieve a 2% Ht. The parasite replication, growth and specific stage of maturity was assessed by examination of a Giemsa-stained (VWR, 352603R) thin blood smear. The parasite cultures were diluted with uninfected O^+ fresh erythrocytes at the trophozoite stage to allow for reinvasion and to obtain the expected parasitaemia levels within the range of 2 – 10%.

2.5.3. Erythrocytes for *P. falciparum* culturing

Whole blood from donors was obtained every two weeks from the SNBTS. The white blood cells were removed by draining the blood through a leucodepletion filter attached to the blood pack, aliquoted into 15 mL or 50 mL Falcon tubes and stored at 4ºC. In some cases, the whole blood was drawn from volunteers in acid-citrate-dextrose tubes (Becton-Dickinson, 366645). In this case, erythrocytes were prepared by diluting the whole blood with an equal volume of incomplete RPMI. Then 10 mL of the diluted blood was layered over 5 mL of Lymphoprep solution (Stemcell Technologies, 07801) in a 15 mL tube. The tube was then centrifuged at 2400 x g speed for 15 minutes, and the supernatant was aspirated. In both cases, the filter erythrocytes were washed twice in 10 mL incomplete RPMI, by centrifugation at 2400 x g for 10 minutes. After the final wash, the erythrocyte pellet was resuspended up to 50% Ht with incomplete RPMI medium for use and stored at 4°C for a maximum of two weeks.

2.5.4. *P. falciparum* Giemsa staining

To determine parasitaemia and assess the different parasite development stages, approximatively 200 μL of the parasite culture was taken every day into an Eppendorf tube

and centrifuged for 30 seconds at 13,000 x g. The supernatant was partially removed; the remaining supernatant was used to resuspend the cells at about 40% Ht and 10 µL smeared at an angle of about 45° on a clean labeled glass slide. The thin smear was air dried with a hair dryer. The slide was fixed with methanol from a sealed glass bottle for at least 20 seconds, then dried again as described above. The slide was stained with freshly made, filtered 10% Giemsa (VWR, 352603R) in Giemsa buffer (Merck, 1.09468) (phosphate buffer tablets in distilled water pH 7.2) for 20 minutes. The slide was rinsed with tap water, dried with a hair dryer, and read under a light microscope (100X objective, with a drop of immersion oil). Parasitaemia was estimated after counting at least 500 erythrocytes in at least 3 random fields. Parasitaemia was defined as the percentage of IEs relative to the total number of erythrocytes counted.

2.5.5. *P. falciparum* ethidium bromide staining

To assess the rosette frequency, an aliquot of 200 µL of the mature pigmented-trophozoite stage culture was transferred into a 1.5 mL Eppendorf tube and incubated with ethidium bromide (Sigma-Aldrich, E1510) (25µg/ mL) for about 2 minutes at 37°C. After incubation, the cells were mixed well by gentle flicking the tube and then 1.4 µL of each stained culture suspension placed onto four spots of a labeled multispot microscope slide (Hendley-Essex, PH-165) and smoothly covered with a coverslip (22 x 22 mm) (Thermo Fisher, 12392108) to gently spread the stained culture suspension over the slide. The slide was immediately visualized with brightfield and uv light at x400 magnification on a Leica DM 2000 fluorescent microscope. A total of 200 IEs were counted to assess rosette frequency (RF) (100 in each spot), with a rosette defined as the ability of an IE to bind two or more uninfected erythrocytes. RF was determined by the proportion of rosettes within the total counted IEs.

2.5.6. *P. falciparum* sorbitol synchronization of ring stage parasites

To establish synchrony of a specific parasite stage, synchronization was carried out with 5% D-Sorbitol solution (Sigma, S-3889) when ring stage parasites were abundant (at least 5% parasitemia) (Lambros & Vanderberg, 1979). Parasite cultures were transferred into a 15 mL Falcon tube, centrifuged at 1000 x g for 5 minutes and the supernatant discarded. The parasite pellet was resuspended in 7 mL of 5% D-Sorbitol and incubated at 37°C for 15 minutes with inversion of the tube at intervals. Only parasitized cells more than ~20 hours post-invasion are permeable to sorbitol. Therefore, they can be lysed and leaving young ring forms and uninfected erythrocytes. After incubation, the mixture was centrifuged and washed twice with

13 mL of incomplete RPMI. The pellet of cells was resuspended in complete medium, gassed, and incubated at 37°C.

2.5.7. *P. falciparum* synchronization of mature knob positive non-rosetting IEs

To obtain synchrony of knob positive IE, cultures with at least 6% parasitaemia at mature stages were concentrated with plasmagel solution (3% succinylated gelatin in normal saline) (Pasvol et al., 1978). Parasite cultures were transferred into a 15 mL Falcon tube, centrifuged at 1000 x g for 5 minutes and the supernatant discarded. The parasite pellet volume was estimated and incomplete RPMI added to obtain a Ht of ~50%. An equal volume of plasmagel was added and mixed, then the tube containing the solution was incubated at 37°C in upright position for 15 minutes or until two separate layers were clearly visible. After incubation, the top layer containing the non-rosetting mature parasites were collected in a new 15 mL Falcon tube, then washed once with 13 mL of incomplete medium and once in 13 mL of complete medium. The parasitaemia was quantified, concentrated mature IE were resuspended in complete medium and fresh uninfected blood were added to obtain 2% Ht, then gassed and incubated at 37°C.

2.5.8. *P. falciparum* cryopreservation

To cryopreserve IEs, parasite culture to be frozen was transferred into a 50 mL Falcon tube and centrifuged at 1000 x g for 5 minutes. Parasite were preserved in glycerolyte solution (45.25 g glycerolyte; 0.1 M sodium lactate ($NaC_3H_5O_3$); 4 mM potassium chloride (KCl); 0.1 M sodium phosphate (NaH_2PO_4); pH 6.8). After centrifugation, the supernatant was aspirated and the pellet resuspended with glycerolyte solution (ratio of 5 volumes of glycerolyte to 3 volumes of packed cells) added dropwise, while gently swirling the tube. After adding the first volume of glycerolyte solution, the cells were allowed to equilibrate for 5 minutes at room temperature (RT) before adding the remaining volumes. The cell suspensions were dispensed into Nunc cryogenic vials (Thermo Fisher Scientific, 366656) and immediately stored at -70ºC freezer for short-term storage and later transferred into liquid nitrogen for long-term storage.

2.5.9. *P. falciparum* thawing

Frozen IEs were thawed at 37°C and transferred into a 50 mL Falcon tube. To 1 mL volume of thawed suspension, 200 µL of sterile 12% NaCl (w/v) was added dropwise and slowly, while gently swirling the tube for at least 2 minutes. The suspension was left to stand at RT for 5 minutes. After which, 10 mL of 1.8% NaCl (w/v) was added dropwise and slowly with gently

agitation, the first 2 mL taking at least 2 minutes. Then, 10 mL of 0.9% NaCl (w/v) and 0.2% glucose (w/v) in the same manner. The suspension was centrifuged at 1000 x g for 4 minutes and the resulting pellet of cells washed with 20 mL of incomplete RPMI. The cells were resuspended in complete RPMI at 2% Ht and 2 drops of fresh erythrocytes added. The flask was gassed and incubated at 37ºC.

2.6. Mycoplasma contamination detection

Parasite and cell cultures were discarded if any contamination (yeast, bacteria, fungi) was observed in the culture flask or through the microscope. Mycoplasma contamination is not visible on Giemsa smears therefore a PCR detection kit (Minerva Biolabs, 11.1100) was used to regularly check for possible contamination. 100 µL aliquot of medium was briefly centrifuged. A total volume of 25 µL per tube was composed of 2 µL of medium, 15.3 µL dH_2O, 2.5 µL primer-nucleotide mix, 2.5 mL 10x buffer (200 mM Tris-HCl pH 8.5, 500 nM KCl, 30 mM $MgCl_2$), 2.5 µL internal control, and 0.2 µL Taq DNA polymerase (Promega, M708B). A PCR reaction was run with the following program: 2 minutes at 94ºC; 35 x (30 s at 94ºC, 30 s at 55ºC, 30 s at 72ºC); and 4ºC forever. A positive control DNA sample and a negative control (H_2O) were used. PCR products were run on a 1% agarose gel. A successful PCR was indicated by a 350 bp (base pairs) product, whereas Mycoplasma positive cultures had an additional band at 280 bp.

2.7. Flow cytometry to detect PfEMP1 expressed by mature IE

PfEMP1 is expressed on the surface of mature IE (pigmented-trophozoites and schizonts). To determine the percentage of IEs exhibiting a specific PfEMP1 variant, it is important to discriminate the mature IE population from the uninfected and ring populations in a mixed culture. To do this, staining was performed with a combination of DNA and RNA dyes as previously described (Ch'ng et al., 2016; Malleret et al., 2011) with Vybrant DyeCycle Violet ™ (VV) (Thermo Fisher Scientific, V35003, 1/2500 final dilution) and Ethidium Bromide (EtBr) (Sigma-Aldrich, E1510, 20 µg/mL final concentration). Vybrant DyeCycle Violet ™ stains DNA, while EtBr stains both DNA and RNA. Both rings and mature pigmented trophozoites possess the same amount of DNA, but mature parasites contain more RNA, allowing their differentiation. Schizonts are distinguished by having increased DNA staining and high RNA staining. The PfEMP1 positive population was detected with staining with primary antibodies against specific PfEMP1 domains (Claessens 2012; McLean 2025; Azazi

refs) and Alexa Fluor™ 647-conjugated anti-rabbit IgG secondary antibody (Thermo Fisher Scientific, A-21245).

Mature IEs (IT4VAR19 and HB3VAR03) with a parasitaemia between 5-10% at 2% Ht were used for flow cytometry analysis. An aliquot of 500 µL of each parasite culture into 1.5 mL Eppendorf tube was centrifuged (5 seconds in a microfuge) and the cell pellets were washed once with 750 µL of PBS/1% BSA. After washing, cell pellets were made up to 2% Ht in PBS/1% BSA and dispensed in 4 aliquots of 49 µL in 1.5 mL Eppendorf tubes labelled as follows (no primary antibody, non-immunized rabbit IgG control, specific PfEMP1 variant antibody, non-specific PfEMP1 variant antibody). The following antibodies (stock are 1 mg/ mL) were added non-immunized rabbit IgG (negative control), no primary antibody (negative control), rabbit 20941 polyclonal anti-IT4VAR19 DBLα IgG1 and rabbit 6358 polyclonal anti-HB3VAR03 DBLα IgG1 at 1 µL in one specific tube to obtain a final concentration of 20 µg/mL in 50 µL.

The samples were incubated on ice for 1 hour and resuspended by gently flicking the tubes every 15 minutes. After incubation, cells were washed twice with 750 µL of cold PBS and 50 µL of the mixture secondary antibody prepared with a total volume of 500 µL PBS/1%BSA + 1 µL Alexa 647-goat anti-rabbit IgG (Thermo Fisher Scientific, A-21245) at 1 µg/ mL in PBS/1%BSA + 0.2 µL of vibrant violet (Thermo Fisher, V35003, 1/2500 final concentration) + 1 µL Ethidium bromide (Sigma -Aldrich, E1510, 10mg/ml stock, 20µg/mL final concentration) was added in each tube and incubated on ice for 45 minutes in the dark and processed as above every 15 minutes. After the second incubation, cells were washed twice with 750 µL of cold PBS, then washed once in 200 µL of PBS/ 0.5% paraformaldehyde (PFA) to fix the cells. The cells were resuspended in 1 mL FACS buffer (PBS/0.1% BSA) in FACS tubes (Falcon 5 mL round bottom polypropylene tubes) (Corning®, 352058) and covered with foil to protect from light. Samples were run on the flow cytometer (Fortessa) as soon as possible, or within 24 hours to preserve the quality of the samples.

2.7.1. Flow cytometry analysis and gating strategy

The acquisition of 100,000 events was analysed using FlowJo software (v.10.8.2). The data was first displayed in a bivariate plot, using side scatter (SSC-A) versus forward scatter (FSC-A). This allowed gating on the erythrocyte population and exclusion of debris (Figure 2-2 A). I then gated to discriminate IEs from uninfected erythrocytes, and to differentiate rings from

pigmented trophozoites and schizonts. Within the erythrocyte population, pacific blue (VV) versus phycoerythrin (PE) (EtBr) was plotted to allow gating on the mature IEs (Vybrant DyeCycle Violet™ high and ethidium bromide high) (Figure 2-2 B). Subsequently, within the mature parasite sub-population, the fluorescence intensity in the APC channel was used to identify antibodies bound to PfEMP1 (Figure 2-2 C). The surface expression level of PfEMP1 was quantified using the median fluorescence intensity (MFI). The MFI data were normalized by dividing the MFI of the test sample by the MFI of the negative control.

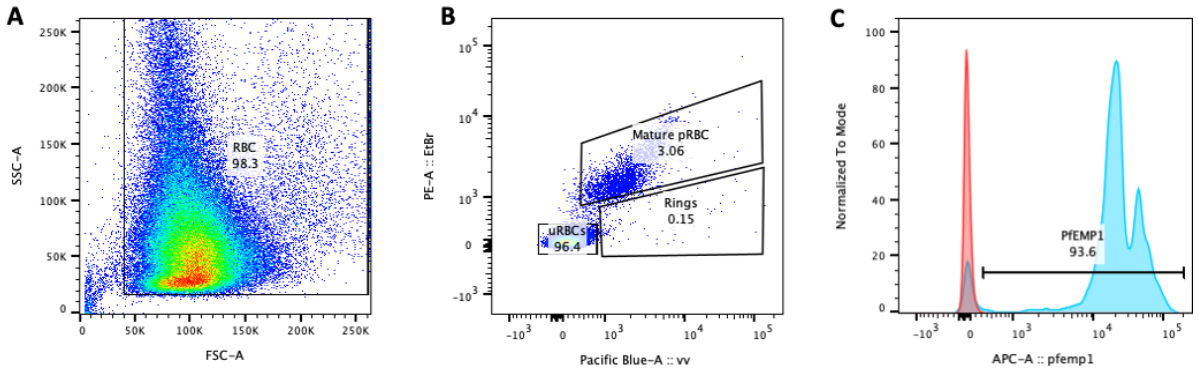


Figure 2-2. Illustration of the gating strategy. (A) RBC (red blood cell)/erythrocyte population is first gated by side scatter (SSA), forward scatter (FSC) to exclude debris. (B) Gating of distinct cell populations: uninfected RBCs/erythrocytes (uRBCs); rings and mature infected erythrocytes (pRBC, i.e., pigmented-trophozoites and schizonts). The discrimination of parasite stages was determined through simultaneous staining with Vybrant DyeCycle Violet™, which stains DNA (pacific blue channel) and ethidium bromide, which stains both DNA and RNA (Phycoerythrin (PE) channel). Mature infected erythrocytes (pigmented-trophozoites and schizonts) have more RNA than rings. (C) IE stained with 20 mg/mL of antibody (e.g. rabbit 20941 polyclonal anti-IT4VAR19 DBLα IgG1) (blue) or an isotype rabbit IgG-negative control (Orange) followed by Alexa Fluor 647-goat anti-rabbit IgG at 1mg/mL were detected and quantified.

2.8. IFA to detect PfEMP1 expressed by mature IEs

The procedure used to stain mature IEs using IFA is like the flow cytometry analysis described above until the end of the primary incubation. After the primary incubation, cells were washed twice with 750 µL of cold PBS. The secondary antibody prepared in a total volume of 500 µL of PBS/1% BSA containing 1 µL of Alexa 488-goat anti-rabbit IgG (Thermo Fisher, A-11034) and DAPI (Molecular, M70277046) at 1µg/mL. 50 µL of the mixture was added to each tube, incubated on ice for 45 minutes in the dark, and resuspended by gently flicking the tubes every 15 minutes. After the incubation time, stained cells were washed three times, twice with 750

µL of cold PBS and once with 750 µL of cold PBS/1% BSA. The cells were resuspended at 40% Ht in PBS/1% BSA and smears were made for each test sample. The smears were air dried and a drop of fluoromount (Sigma, F4680) was added to each smear and covered with a 22 x 22 mm coverslip. Nail varnish was used to seal edges of the coverslip and smears were stored at 4°C overnight and viewed the next day with a fluorescence microscope (Leica, DM LB 2) using a 100X objective. The percentage of DAPI positive IEs with punctate fluorescence of PfEMP1 staining out of 100 erythrocytes in a minimum of 4 fields were scored.

2.9. Selection by panning of *P. falciparum* on hCMEC/D3

Culture-adapted IEs usually only bind at low level to HBECs *in vitro*, unlike the high-density IE sequestration described in postmortem cerebral malaria cases (MacPherson et al., 1985; Milner et al., 2015; Seydel et al., 2006). Therefore, it is important to select various IE strains for adhesion to HBECs to increase the IE population's binding capabilities (Claessens & Rowe, 2012). The selection process, known as the panning method, is carried out by incubating IEs with HBECs as described below.

Cytoadherence assays with hCMEC/D3 were performed in 60 x 15 mm petri dishes (BD, 351005) coated with 1% of collagen in PBS. Cells in complete DMEM/F-12 medium were grown in each petri dish to reach 80- 90% confluency after 48 or 72 hours. The day of the assay, dishes, and mature IEs (5- 10% parasitemia and 30 µL packed cell volume) were washed twice with incomplete DMEM binding medium (bicarbonate-free DMEM/ F-12 Ham with L-glutamine and 15 mM Hepes (Sigma, D8900)). Then 1.5 mL of IE resuspended in complete binding medium (1.5 mL of incomplete DMEM bicarbonate-free medium plus 1% BSA per dish) at 2% Ht were added in a dish containing a layer of confluent hCMEC/D3. Dishes were incubated at 37ºC for 75 minutes with gentle agitation after 30 and 60 minutes to resuspend non-adherent cells. Unbound IE and uninfected erythrocytes were removed by gentle hand washing (at least 5 washes) with 3 mL of incomplete binding medium per wash and the monitoring of adherent cells was carried out by viewing with an inverted microscope. After washing, fresh uninfected erythrocytes were added to the few IE adhering to hCMEC/D3, dishes were placed in a gassed parasite culture chamber and incubated overnight at 37ºC. Schizont stage parasites burst, merozoites reinvaded uninfected erythrocytes and these ring stage parasites were collected the following day. These parasites were washed once in 10 mL incomplete medium, resuspended in 5 mL complete culture medium, gassed for 15 seconds, and incubated at 37 ºC. Parasites were cultured till enough mature stage parasites are obtained

(between 3 to 4 weeks and less after 2 to 3 rounds of selection) and a new round of selection can be carried out.

2.10. Static adhesion assays to receptor molecules immobilised on plastic

To identify specific receptors used by mature IE to interact with hCMEC/D3, I carried out static binding assays using soluble proteins absorbed onto plastic. A maximum of twelve circles were drawn on the back of the 60 x 15 mm untreated plastic petri dishes (BD, 351005). 3 µL of recombinant proteins (Table 2-4) were spotted in duplicate and in a radial manner at an equal distance from the centre, and at a concentration of 50 µg/mL in PBS per receptor: CSA, integrin αvβ3, TSP, VCAM-1, ICAM-1, EPCR, C1QBP, CD31, and 25 µg/mL for CD36 used as positive control, and PBS as negative control. Dishes were incubated in a humidified chamber overnight at 4ºC to allow proteins to adsorb on the plastic surface. The next day, the soluble protein solutions were aspirated off using a P1000 pipette tip attached to a vacuum pump, and dishes were blocked for 2 hours at 37ºC with PBS/2% BSA called "blocking buffer". After the blocking step, dishes and IEs (5-10% parasitemia) were washed twice in incomplete binding medium (bicarbonate-free RPMI 1640 containing glucose, Hepes and L-glutamine (Gibco, 13018-031)) prior to adding 1.5 mL of IE resuspended in complete binding medium (incomplete bicarbonate-free RPMI medium plus 1% BSA) at 2% Ht. Dishes were incubated at 37ºC for 75 minutes with gentle resuspension every 12 minutes. Unbound infected and uninfected erythrocytes were removed by gentle hand washing (at least 5 washes) with 3 mL of incomplete binding medium per wash (checking of adherent cells was carried out with an inverted microscope). Adherent IEs were fixed using PBS/2% glutaraldehyde for at least 30 minutes, followed with extensive washing with tap water, and stained with 5% Giemsa for 10 minutes. IE binding levels were quantified with an inverted microscope (Yenway, XD30) at 40X, and results were expressed as the number of IE bound per spot (randomly count in at least 3 fields for each radial spot). In each experiment, two identical dishes were used per parasite line.

Table 2-4. Recombinant proteins and biomolecules used in spot binding assays

Candidate proteins	Supplier, Catalog number	Type	Stock concentration	Test concentration	In 15µl
CD36	R&D System, 1955-CD	Recombinant	100 µg/mL	25 µg/mL	3.75µL + 11.25µL of 1xPBS
C1QBP	Sino Biological, 11874	Recombinant	1 mg/mL	50µg/mL	0.75µL + 14.25µL of 1xPBS
TSP	Calbiochem, 605225	Purified	250 µg/mL	50 µg/mL	3µL + 12µL of 1xPBS
ICAM1	R&D System, 720-IC	Recombinant	200 µg/mL	50 µg/mL	3.75µL + 11.25µL of 1xPBS
PECAM1	R&D System, ADP6	Recombinant	330 µg/mL	50 µg/mL	2.3µL + 12.7µL of 1xPBS
VCAM1	R&D System, 862-VC	Recombinant	50 µg/mL	50 µg/mL	
EPCR	Sino Biologicals, 13320-H02H	Recombinant	250 µg/mL	50 µg/mL	3µL + 12µL of 1xPBS
Integrin αVβ3	Chemicon, AG265	Purified	215 µg/mL	50 µg/mL	3.5µL + 11.5µL of 1xPBS
CSA	Sigma, 27042	Bovine trachea	1 mg/mL	50 µg/mL	0.75µL + 14.25µL of 1xPBS

2.11. Staining of bound IE from static adhesion assay

To determine whether IEs interacting with specific receptors immobilised on plastic dishes are expressing the PfEMP1 variant of interest, I stained bound IE with rabbit polyclonal antibodies to IT4VAR19 DBLα. The static adhesion assay was performed as above until the end of the washing steps. Dishes were rinsed once with 3 mL of PBS and spots were delimited with the IFA pen (PAP Pen, DAI-PAP-S), then 20 µL of 20 µg/mL rabbit polyclonal IgG to IT4VAR19 DBLα in PBS/1%BSA was added on each spot and allowed to incubate for 30 minutes in a humid box at 37ºC. After this incubation step, dishes were washed with 5 mL of PBS, and the wash was removed with a plastic Pasteur pipette. Another 5 mL of PBS was then added and left for 5 minutes. The wash solution was then removed as above, and 50 µL of 1:500 Alexa-488 goat anti-rabbit IgG secondary antibody in PBS/1% BSA plus 1µg/mL of DAPI was added on each spot and allowed to incubate for 30 minutes in a humid box at 37ºC. After this step, dishes were washed as above. The wash solution was removed, and a drop of fluoromount (Sigma, F4680) and round coverslip was added to each spot. Dishes were stored at 4ºC in the dark and viewed the next day under fluorescence microscopy with a 100X objective. Images were processed, analysed and saved as described above in section 2.3.

2.12. Static HBEC-binding assay, inhibition assays with antibodies and recombinant proteins

To test the inhibitory effect of antibodies and recombinant proteins, hCMEC/D3 cells were grown with 10% normal human plasma to allow soluble C1QBP to associate with the cell surface membrane in 1% collagen precoated 30 x 15 mm petri dishes (BD, 3100). The day of the assay, confluent hCMEC/D3 complete medium was removed, cells were washed twice with 2 mL incomplete medium or PBS. Resting hCMEC/D3 cells were incubated with 50 µg/mL mouse monoclonal and rabbit polyclonal antibodies to human C1QBP (Sino biological, MM09 and RP01 respectively), 30 µg/mL of rat monoclonal antibody to human EPCR (Hycult, HM2145), or 20 µg/mL of mouse monoclonal antibody to human ICAM1 (Santa Cruz Biotechnology, 107) in 400 µL of complete cell culture medium for 1 hour at 37ºC with smooth agitation after 30 and 60 minutes to resuspend non-adherent antibodies. To test the inhibitory effect of recombinant proteins, mature IEs were resuspended at 2% Ht with 50 µg/mL of soluble C1QBP (Sino Biological, 11874), 20 µg/mL of soluble EPCR (Sino Biological, 13320-

H02H), or 20 μg/mL of soluble ICAM1(R&D system, 720-IC) in 500 μL of IE complete culture medium for 1 hour at 37ºC.

In both cases, after incubation, the complete medium was removed without washing step and the binding assay performed as described in section 2.9, with 500 μL of IEs in binding medium at 2% Ht per dish (Azasi et al., 2018). Dishes containing only confluent and resting hCMEC/D3, with binding medium only considered as control dishes.

After washing, adherent IE cells were fixed with PBS/2% glutaraldehyde (Sigma, G6257) for at least 30 minutes, then washed extensively with tap water (to remove excess of glutaraldehyde, avoid masking of surface cellular components, and reduce background interference) and stained with 5% Giemsa for 10 minutes. IE binding levels were quantified with an inverted microscope at 40X, and results were expressed as the number of IE bound per 100 hCMEC/D3 (Azasi et al., 2018). All cytoadherence assays were carried out in duplicate (i.e. two dishes per condition). Images were acquired with a DFC300FX digital camera fixed to the fluorescence microscope (Leica, DM LB 2). Images were processed, analysed and saved as described above in section 2.3.

2.13. Statistical analyses

Statistical analyses and graphing of binding assay data were done using GraphPad Prism version 9.5.1. For each binding assay type, at least 3 independent experiments were carried out on different days to provide biological replicates (n=3 or more in all analyses). Within each independent experiment there were technical replicates (duplicate spots, dishes or wells which were averaged to give a single value for each condition in each experiment). Raw data were Log transformed to normalize the data distribution. For comparison between the means of two groups, a two-tailed t test with Holm-Sidak multiple comparisons test was used. For comparison between the means of three or more groups, an ordinary one-way ANOVA was used with Dunnett's posthoc test. P values ≤ 0.05 will be significant, asterisks indicate *P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001, and ns indicates no statistical significance (P > 0.05).

3. Chapter III: Investigation of C1QBP expression by human endothelial cells

3.1. Abstract

CM is characterised by the massive adhesion of IEs in the human brain microvasculature. IEs have been shown to interact with HBEC *in vitro*. In a prevous study, this adhesion phenotype was reduced by a monoclonal antibody against C1QBP and recombinant C1QBP in dose-dependent fashion. These results suggested C1QBP is a potential host endothelial cell receptor for IE cytoadherence during CM. However, despite this adhesive interaction being described, the cellular localisation of host C1QBP remains a matter of debate in the literature. The main aims of this chapter were to identify the cellular localisation of C1QBP using an immortalised HBEC line, hCMEC/D3, and to determine whether the same staining pattern applied to other human endothelial cells (HBEC-5i, primary HBMEC, primary HPMEC, and primary HDMEC). The cellular localisation of C1QBP as well as other putative receptors on hCMEC/D3 was investigated using IFA and fluorescence microscopy. Both immortalised HBEC lines (hCMEC/D3 and HBEC-5i) in resting and TNFα-activated conditions showed intracellular staining for C1QBP, but cell surface staining was not observed. However, after preincubation with soluble C1QBP, both immortalised lines did exhibit positive surface membrane expression of C1QBP in resting and activated conditions. In contrast, primary HBECs from brain, lung and skin (HBMEC, HPMEC, and HDMEC) showed constitutive positive surface membrane staining for C1QBP. hCMEC/D3 cells also displayed positive surface membrane staining for other known *P. falciparum* adhesion receptors including ICAM1, VCAM1, PECAM1 and EPCR in both conditions. Whereas other putative IE receptors such as CD36, CSA, NCAM, fractalkine, thrombospondin, selectin E and selectin P were not detected. These findings highlighted that C1QBP is constitutively expressed on the surface of primary HBECs , and could therefore play a role in sequestration during CM. The immortalised cell lines used for *in vitro* studies do not express surface C1QBP initially, but it can become associated with the cell surface from human plasma or if added exogenously. These findings suggest the importance of validating immortalised human cell lines used for cytadhesion research by comparing expression of receptors of interest with the relevant primary human endothelial cells.

3.2. Introduction

The characteristic feature of *P. falciparum* infection is the sequestration of mature forms of IEs within the microvasculature of major organs of the body, such as the brain, gastrointestinal tract, heart, lung, kidney and skin (MacPherson et al., 1985; Milner et al., 2015). Sequestration involved molecular interactions between antigens located on the surface of IEs and host receptors expressed on the surface of endothelial cells (Rowe et al., 2009). In CM, the sequestration of IEs leads to microvascular obstruction, metabolic acidosis, hypoxia, coma and often death (Miller et al., 2002). IE cytoadherence can also trigger an inflammatory response in the brain, leading to tissue damage (Zougbede et al., 2011). Soluble mediators such as cytokines are known to regulate adhesion molecule expression and thus may modulate cytoadherence (Xiao et al., 1996; Zougbede et al., 2011).

The sequence of events leading to CM, starting either with the accumulation of IEs in the brain microvasculature and/or with the rupture of mature IEs and the release of parasite-derived toxins (Zougbede et al., 2011) can only be determined by *in vivo* approach. However, ethical and cultural constraints have hampered the detailed investigation of the pathophysiology of human CM. Researcher have therefore developed three major approaches to define the pathogenesis of CM. Firstly, postmortem studies of CM are limited to histopathological analysis of fatal CM cases with non-CM cases (Dorovini-Zis et al., 2011; MacPherson et al., 1985; Taylor et al., 2004). These studies demonstrate the direct interaction between IE and the host endothelial cells. Meanwhile, postmortem studies cannot describe the natural history of the disease or compare fatal cases with those who survive after treatment. The difficulties in obtaining postmortem samples due to social and cultural reasons are also evident barriers to undertake this type of study. Secondly, the mouse model (also called “experimental cerebral malaria”) is widely used due to many advantages. This model allows study of the parasite development stages from infection to transmission and the dissection of molecular interactions that are difficult to study in humans (de Souza et al., 2010). However, *P. falciparum* is totally dependent to Homo sapiens and is unable to infect rodents. Therefore, the rodent models use malaria parasites such as *P. chabaudi*, *P. berghei* or *P. yoelli*. Hence this model does not perfectly resemble human disease.

Thirdly, *in vitro* models have been used to perform cytoadhesion studies. These are based on diverse cell types including HUVEC (human umbilical vein endothelial cells) (Udeinya et al.,

1981), C32 amelanotic melanoma cells (Schmidt et al., 1982), CHO (Chinese hamster ovary cells) (Oquendo et al., 1989), COS-7 cells (Udeinya, 1990), and HDMEC (Siano et al., 1998). These cells do not originate from human brain endothelial cells, but they can interact with IE. To overcome this limitation, more relevant endothelial cells from human brain origin have been developed and used, such as immortalised HBECs, HBEC-5i (Dorovini-Zis et al., 1991), and hCMEC/D3 (Weksler et al., 2005). These human endothelial cell lines can be frozen, thawed, expanded many times and maintained their viability, adhesiveness and particular endothelial cell features such as showing positive intracellular staining to vWF and negative staining for SMA. Mature IE adhere specifically to these cultured HBEC (Avril et al., 2013; Avril et al., 2012; Claessens et al., 2012). Key findings from studies of cytoadherence using immortalised and primary HBEC are contributing to our understanding of the molecular basis of CM (Avril et al., 2012; Azasi et al., 2018; Claessens et al., 2012). Therefore, the use of *in vitro* endothelial cell models constitutes a suitable alternative to investigate the molecular pathogenesis of CM.

Previous studies claim C1QBP as an important receptor for IE adhesion during severe malaria (Biswas et al., 2007; Magallon-Tejada et al., 2016; Mayor et al., 2011). However, conflicting evidence exist about the cellular localisation of C1QBP (Dedio et al., 1998; Dedio & Muller-Esterl, 1996; Eggleton et al., 1995; Ghebrehiwet et al., 1994; Peerschke et al., 1996). This thesis chapter aims to determine the cellular localisation of host C1QBP using human endothelial cells from diverse tissues.

3.3. Objectives of this chapter

This chapter will aim:

1. To determine whether C1QBP is constitutively expressed by human endothelial cells using two types of endothelial cells:
    a) Immortalised human brain endothelial cells (hCMEC/D3 and HBEC-5i)
    b) Primary human endothelial cells (HBMEC, HPMEC, and HDMEC)
2. To characterise the known *P. falciparum* receptors expressed by hCMEC/D3
3. To test whether recombinant C1QBP is adsorbed onto the surface of hCMEC/D3
4. To determine if soluble C1QBP is detectable in normal human plasma, and plasma from patients infected with *P. falciparum*

3.4. Methods

Routine HBEC culture, IFA and fluorescence microscopy (Figure 3-1), and ELISA approaches are described in chapter 2.

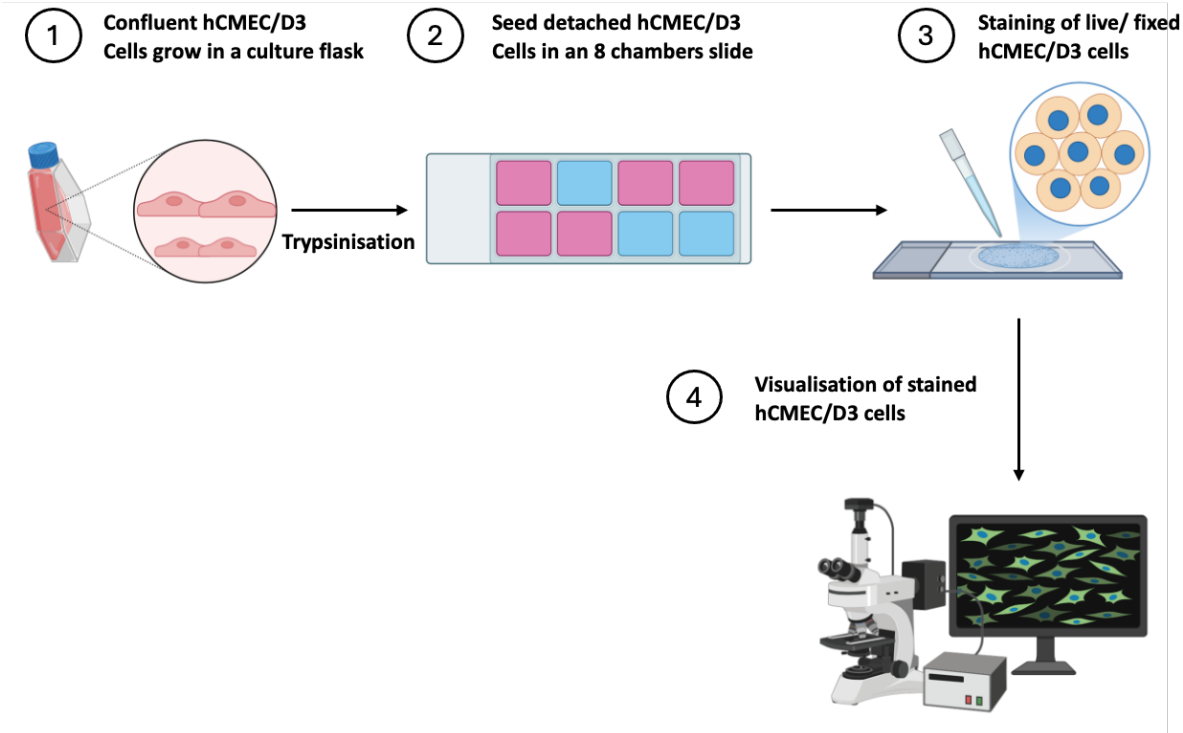


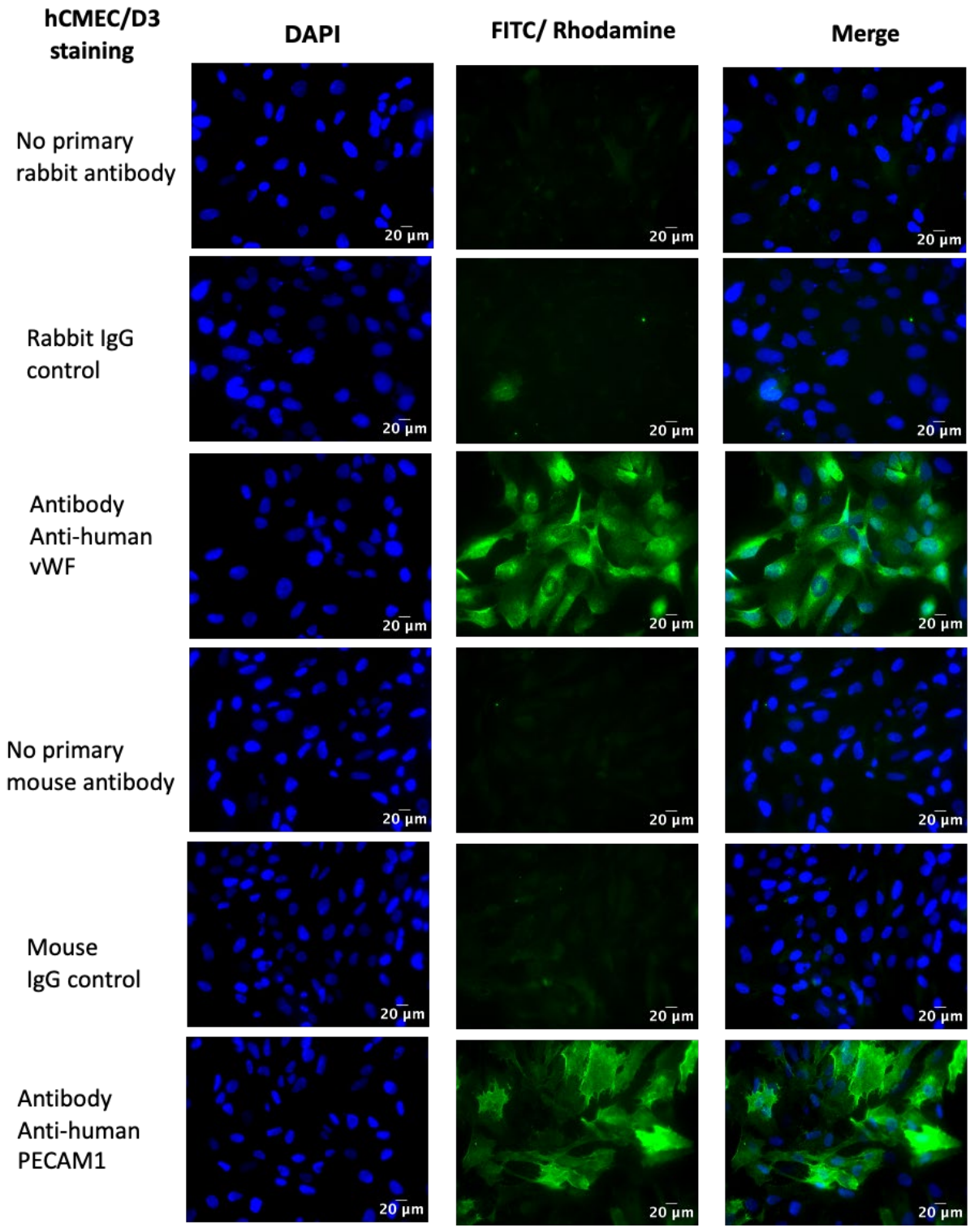
Figure 3-1. Illustration of hCMEC/D3 surface receptors characterisation using IFA. hCMEC/D3 cells are grown in a culture flask. Confluent Cells are detached by trypsinisation and reseeded in an 8 chambers slide. Adherent hCMEC/D3 cells are fixed and stained with specific antibody anti-human C1QBP/other receptors. Stained hCMEC/D3 cells are visualise using a fluorescence microscope. The diagram is generated with BioRender.com.

3.5. Results

To determine the cellular localisation of host C1QBP, I used an immortalised HBEC line isolated and characterised to exhibit human endothelial cell nature, termed hCMEC/D3 (Weksler et al., 2005). Few studies have used hCMEC/D3 to elucidate mechanisms of IE cytoadherence (Jambou et al., 2010; Zougbede et al., 2011). Firstly, I authenticated the endothelial cell identity of hCMEC/D3 by staining with antibodies to endothelial cell markers. I then characterised the cellular localisation of C1QBP, and other potential receptors known to play a role in IE sequestration. I determined the presence/absence of positive immunofluorescent staining with specific antibodies for human endothelial cell receptors visually, and compared to suitable isotype controls such as rabbit and goat polyclonal antibodies, and mouse and rat monoclonal antibodies.

3.5.1. C1QBP is not constitutively expressed by hCMEC/D3 in immunofluorescence assays.

To authenticate the endothelial origin of hCMEC/D3, the cells were labelled with antibodies for human endothelial cell markers including vWF, PECAM1/ CD31, Dil-Ac-LDL, and a negative control SMA which is a marker for fibroblasts. Fixed and permeabilised resting hCMEC/D3 were incubated with rabbit polyclonal antibody to human vWF. The staining showed positive intracytoplasmic granules signal compared to negative controls, which did not show any signal (Figure 3-2, rows 1, 2 and 3 from the top). Fixed hCMEC/D3 cells were stained with mouse monoclonal antibody to human PECAM1/ CD31 and to human SMA. The hCMEC/D3 cells surface membrane showed positive immunofluorescent staining for the endothelial marker PECAM1/CD31, unlike to their negative controls (Figure 3-2, rows 4, 5 and 6 from the top), while the anti-human SMA did not show any immunofluorescence (Figure 3-2, rows 4, 5 from the top and row 3 from the bottom). The PECAM1/CD31 staining is a good example of the appearance of surface membrane staining, with green fluorescence over the entirety of the endothelial cell body and a clear cell outline (Figure 3-2, row 6 from the top). In the presence of Dil-Ac-LDL in the culture medium, live hCMEC/D3 cells internalised the Dil-Ac-LDL, which was seen as red punctuate particles with a perinuclear distribution (Figure 3-2, row 1 from the bottom). These characteristics did not appear in the absence of Dil-Ac-LDL in the hCMEC/D3 cell culture medium (Figure 3-2, row 2 from the bottom). Collectively, these results validate the human endothelial nature of hCMEC/D3 in this study and confirm that there is no fibroblast contamination of this endothelial cell line.



Continued next page

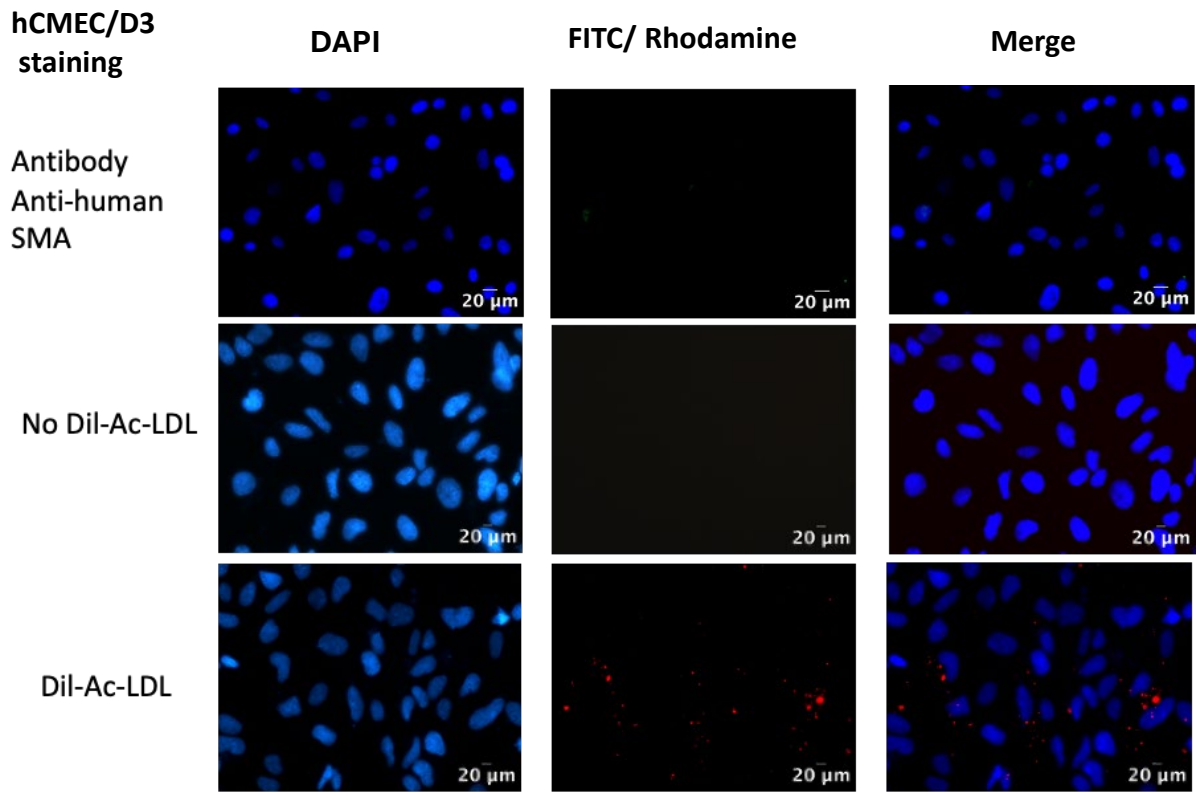
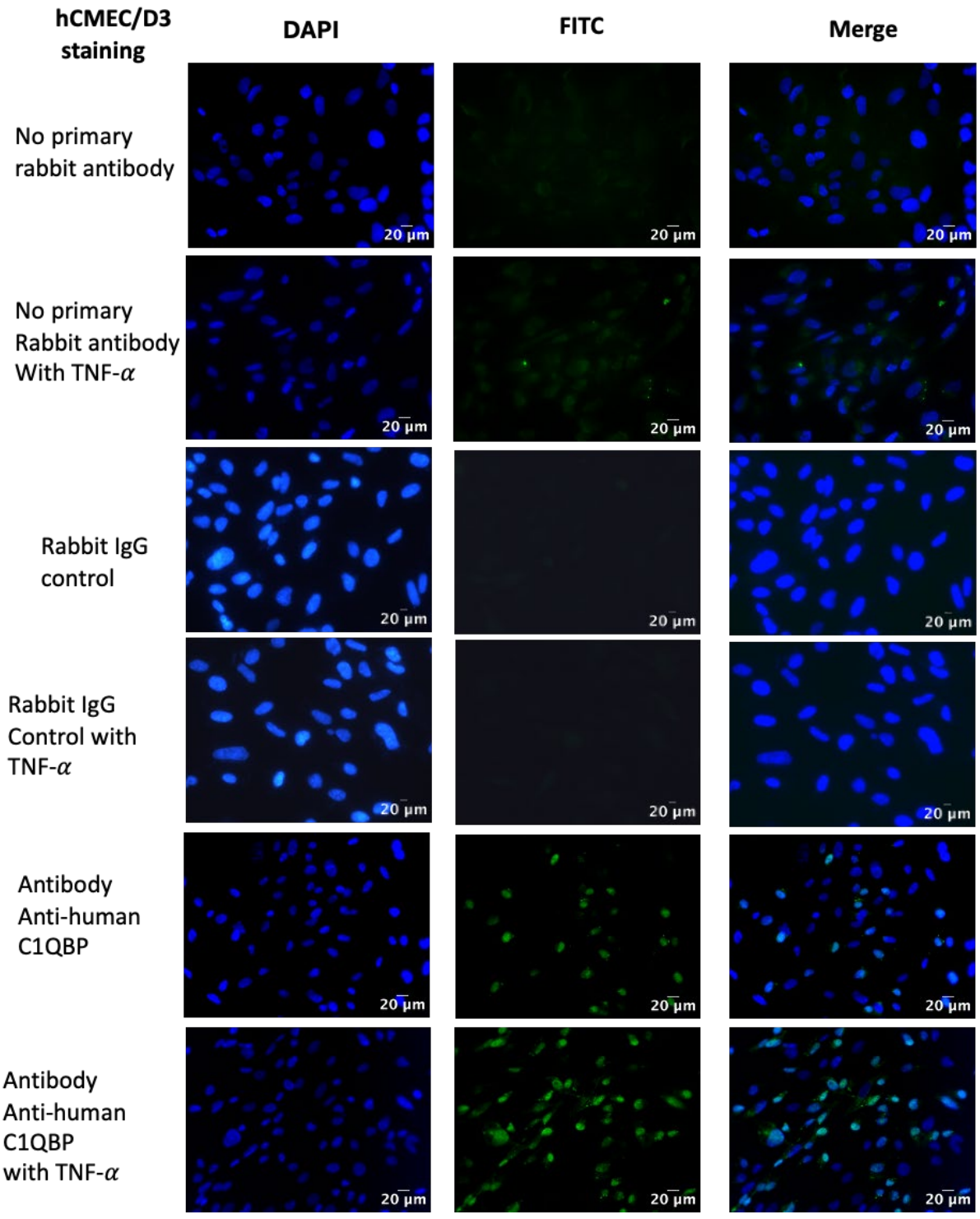


Figure 3-2. Authentication of hCMEC/D3 as a human endothelial cell line. hCMEC/D3 cells were fixed with 1% paraformaldehyde, blocked with 5% goat serum then stained with primary antibody according to manufacturer's instructions for vWF, PECAM1, SMA, and their respective negative controls. Immunofluorescence was detected by an appropriate secondary isotype antibody Alexa fluor 488 conjugated and read with FITC filter (green) and counterstained the cell nuclei with DAPI (Blue). Live hCMEC/D3 were incubated with Dil-Ac-LDL and without Dil-Ac-LDL (negative control) in the cell culture medium for 4 hours at 37°C, then fixed with 1% paraformaldehyde and stained the cell nuclei with DAPI (Blue). The uptake of Dil-Ac-LDL by hCMEC/D3 was read with rhodamine filter (red) and the cell nuclei with DAPI (Blue). Independent experiments were conducted at least twice for each marker and the images shown are representative. All pictures were taken at x400 magnification with a scale bar of 20µm.

I further performed IFA on resting, TNFα-activated, and fixed hCMEC/D3 cells to determine the precise cellular localisation of C1QBP and other known endothelial cells receptors involved in IE sequestration such as ICAM1, CD36, VCAM1, selectin E and P, Fractalkine, CSA, NCAM, integrin αvβ3, TSP, PECAM1/CD31 and EPCR. Some of these endothelial cell surface membrane proteins can be upregulated/ dysregulated by pro-inflammatory cytokines like TNF-α. TNF-α is a cytokine that has been incriminated to play a role in the pathogenesis of CM (Kwiatkowski, 1990).

Resting, TNF-α activated, and fixed hCMEC/D3 cells surface membrane were incubated with rabbit and goat polyclonal antibodies to human C1QBP, human integrin αvβ3, and human fractalkine respectively (Figure 3-3). Resting and TNF-α activated hCMEC/D3 cells do not show surface membrane staining for C1QBP. The cells display intracellular peri-nuclear immunofluorescent staining for C1QBP in both conditions (resting and activated) compared to their negative controls (Figure 3-3, rows 1 to 6 from top). hCMEC/D3 cells do not exhibit positive surface membrane staining, but cells show peri-nuclear staining for integrin αvβ3 in resting and activated conditions, while their negative controls show only background staining (Figure 3-3, rows 1 to 4 from top and rows 3 to 4 from the bottom). Resting and TNF-α activated hCMEC/D3 cells did not display any staining for fractalkine, appearing like their negative controls (Figure 3-3, rows 1 to 4 from top and rows 1 to 2 from bottom).



Continued next page

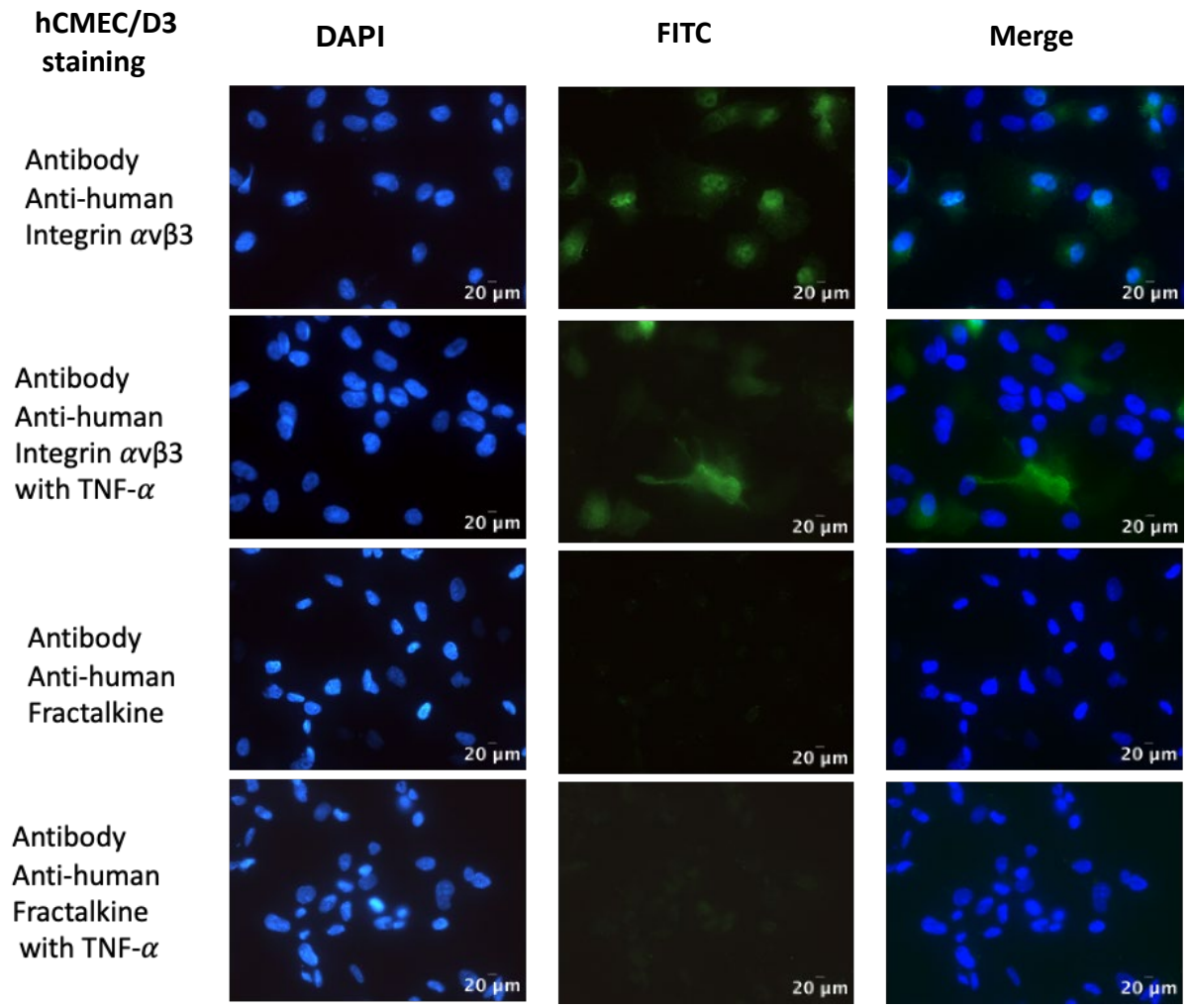
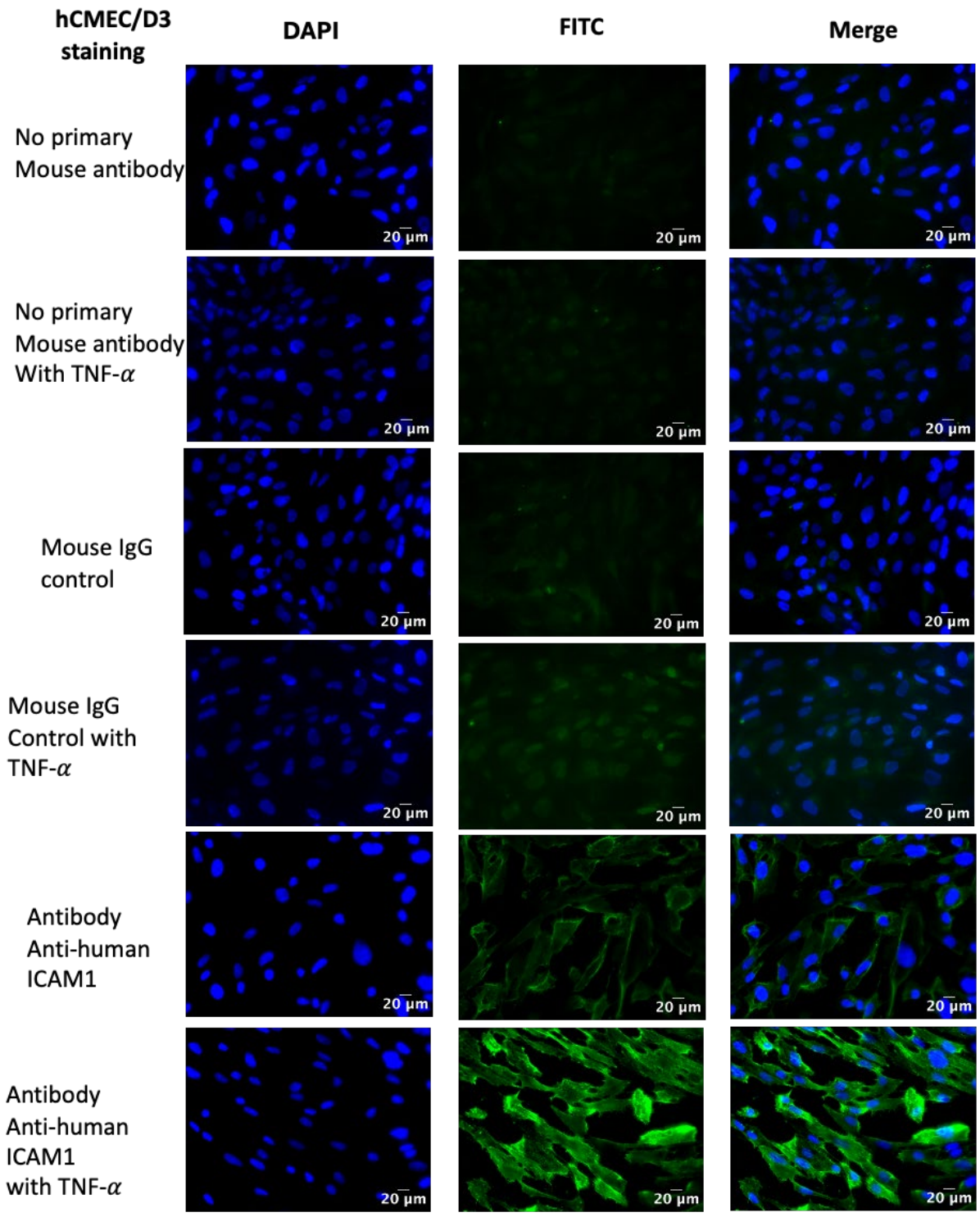


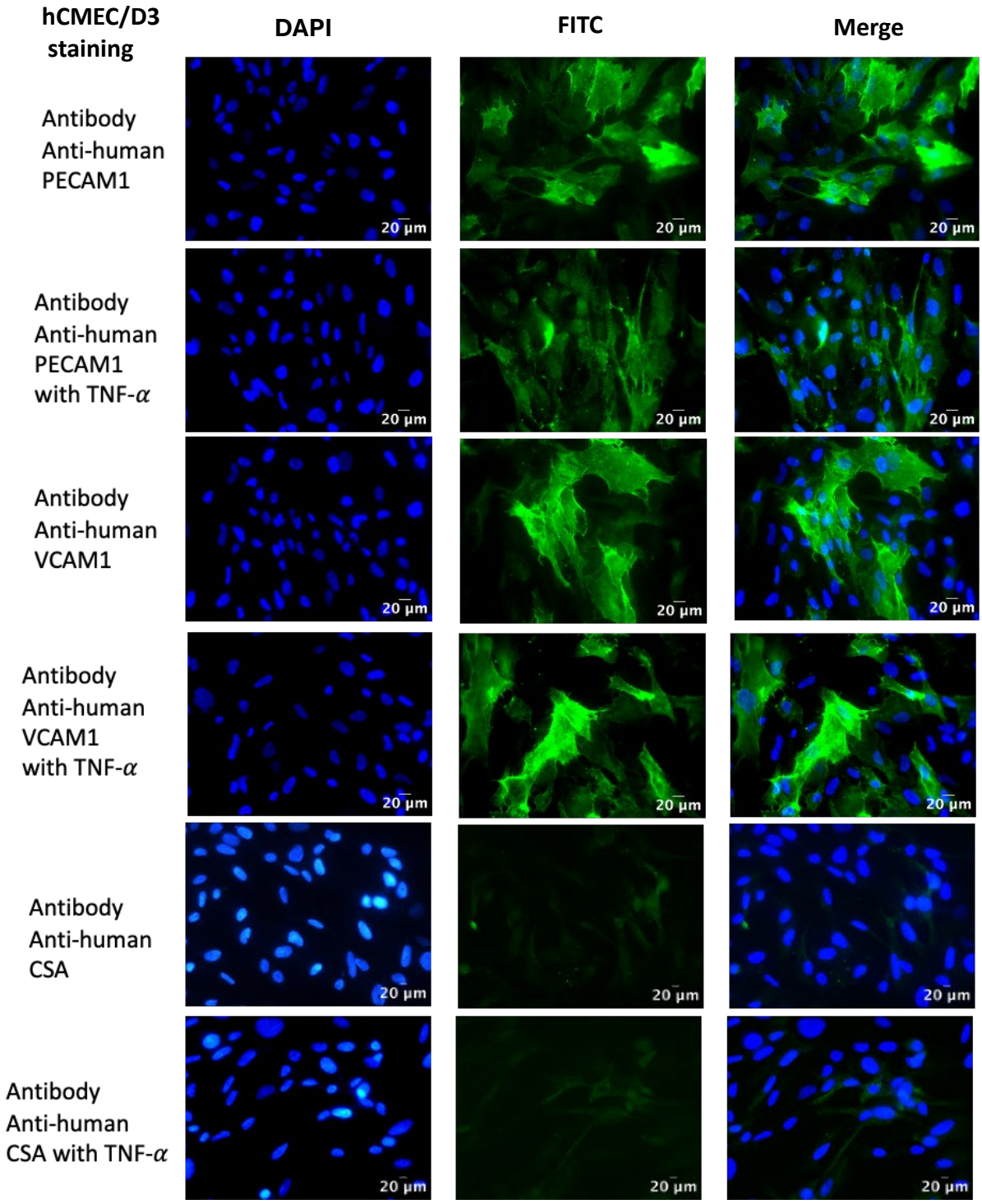
Figure 3-3. Characterisation of hCMEC/D3 with rabbit and goat polyclonal antibodies. Rabbit antibodies investigated were against human C1QBP and integrin αvβ3. Goat antibodies studied were against human fractalkine. In both conditions, cells were fixed with 1% paraformaldehyde, blocked with 5% goat serum then stained with primary antibody according to manufacturer's instructions. Immunofluorescence was detected by an appropriate secondary antibody Alexa fluor 488 conjugated and read with FITC filter (green) and counterstained the cell nuclei with DAPI and read with DAPI filter (Blue). Independent experiments were conducted at least twice for each candidate receptors and the images shown are representative. All pictures were taken at x400 magnification with a scale bar of 20µm.

Resting and TNF-α activated fixed hCMEC/D3 cells were stained with mouse monoclonal antibodies to human ICAM1, PECAM1/CD31, VCAM1, CSA, CD36, NCAM, TSP, selectin E and P respectively (Figure 3-4). In both conditions (resting and activated), hCMEC/D3 cells show positive immunofluorescent surface membrane staining for ICAM1, PECAM1/CD31, and VCAM1 differently from their negative controls (Figure 3-4, rows 1 to 12 from the top). Whereas CSA, CD36, NCAM, TSP, selectin E and P selectin do not show any clearly positive fluorescent signal and are comparable to their negative controls (Figure 3-4, rows 1 to 10 from

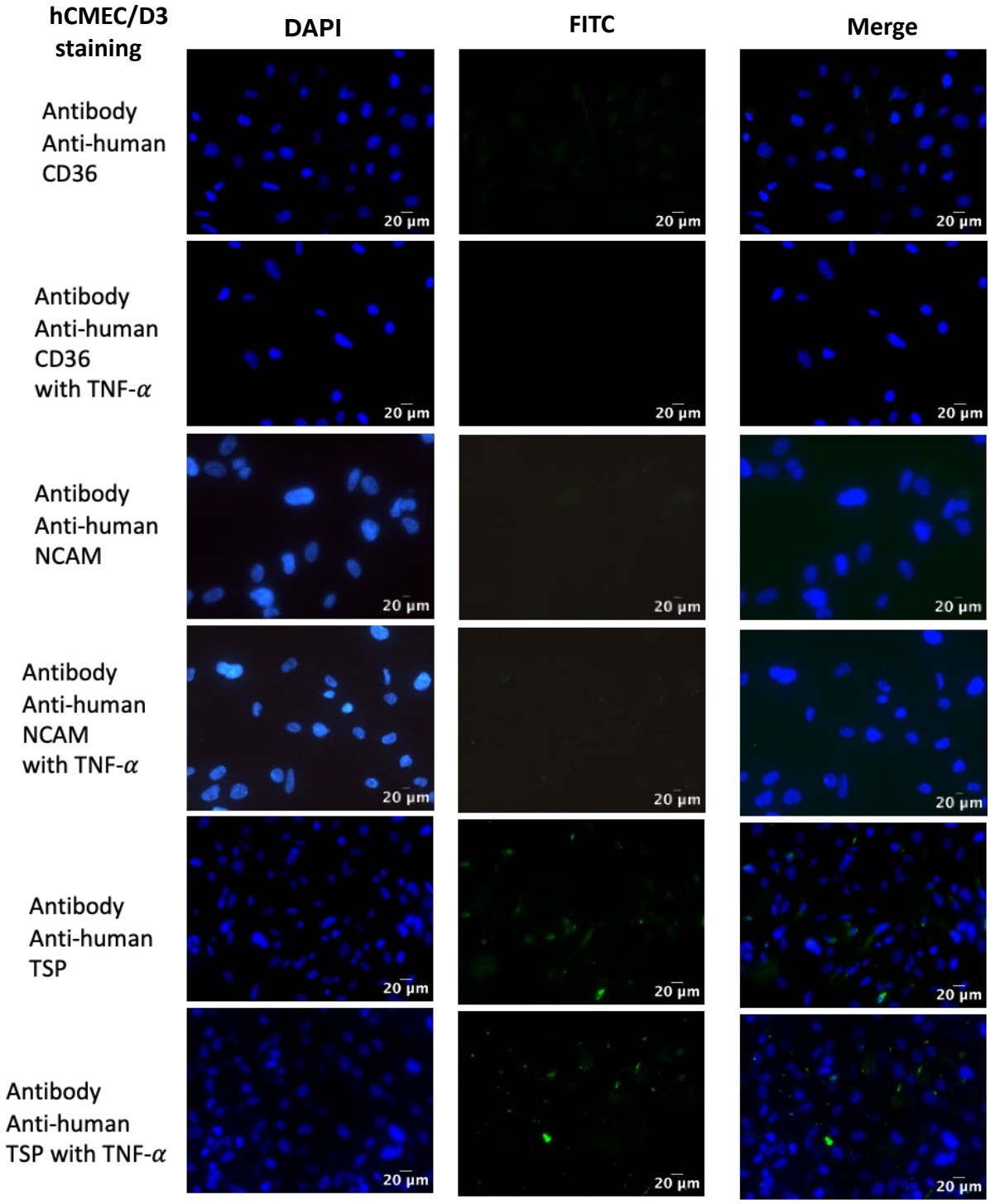
the bottom and rows 1 to 4 from the top). Activation of hCMEC/D3 cells with TNF-α results in an increase of ICAM1 expression (Figure 3-4, rows 2, 4 and 6 from the top), but has no marked effect on staining for any other receptor (Figure 3-4).



Continued next page



Continued next page



Continued next page

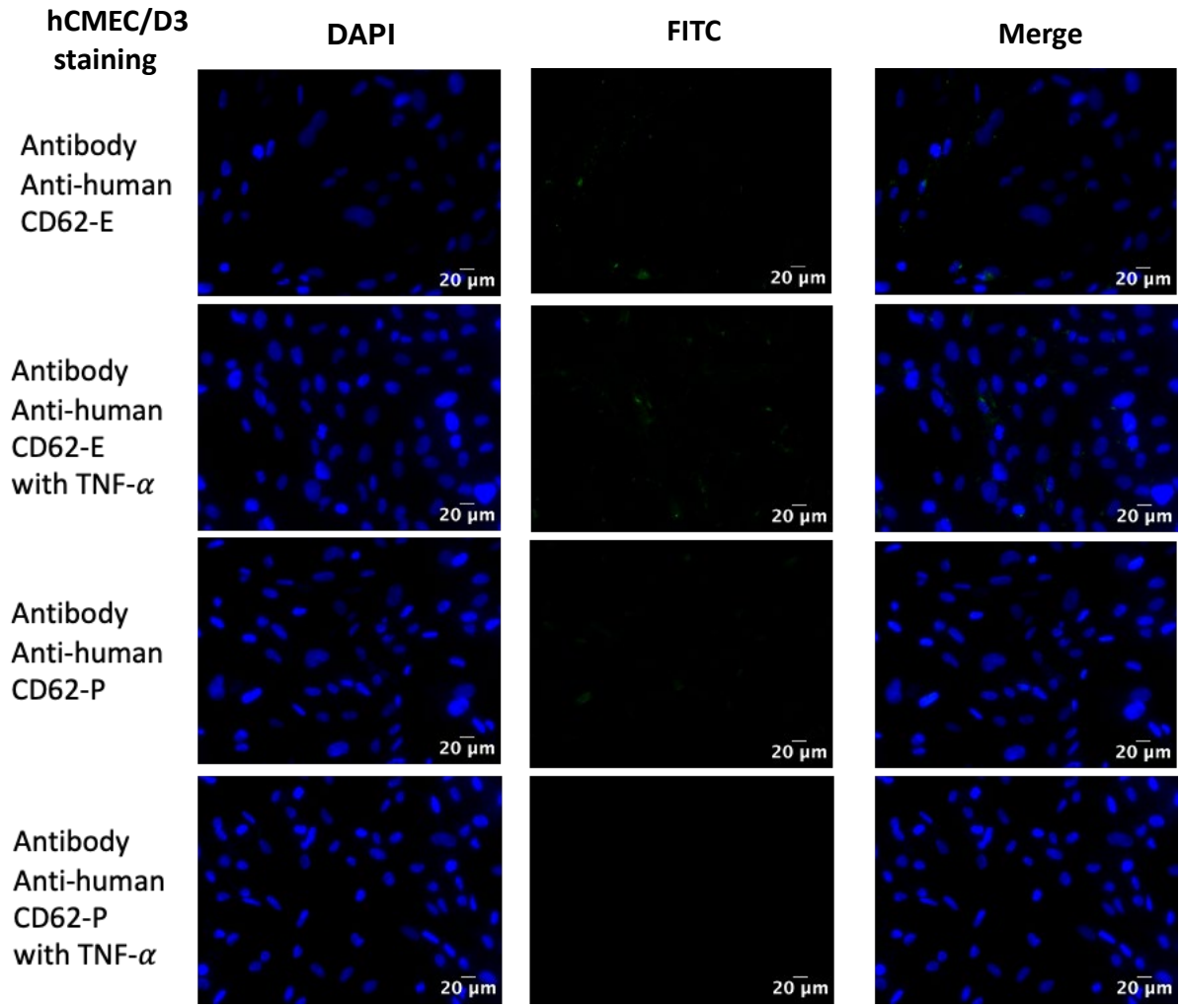


Figure 3-4. Characterisation of hCMEC/D3 with mouse monoclonal antibodies. Proteins assessed were ICAM1, PECAM1, VCAM1, CSA, CD36, NCAM, TSP, E and P selectin. In resting and activated conditions, hCMEC/D3 cells were fixed with 1% paraformaldehyde, blocked with 5% goat serum then stained with primary antibody according to manufacturer's instructions. Immunofluorescence was detected by an appropriate secondary antibody Alexa fluor 488 conjugated and read with FITC filter (green) and counterstained the cell nuclei with DAPI and read with DAPI filter (Blue). Independent experiments were conducted at least twice for each candidate receptors and images shown are representative. All pictures were taken at x400 magnification with a scale bar of 20µm.

Resting and activated hCMEC/D3 cells surface were incubated with rat monoclonal antibody to human EPCR (Figure 3-5). In both conditions, hCMEC/D3 cells displayed positive immunofluorescent staining for EPCR distinct to their negative controls (Figure 3-5, rows 1 to 6 from top). However, activated hCMEC/D3 cells resulted in a decrease of EPCR expression

(Figure 3-5, rows 2, 4 and 6 from the top) as has been noted previously (Simmonds & Lane, 1999) .

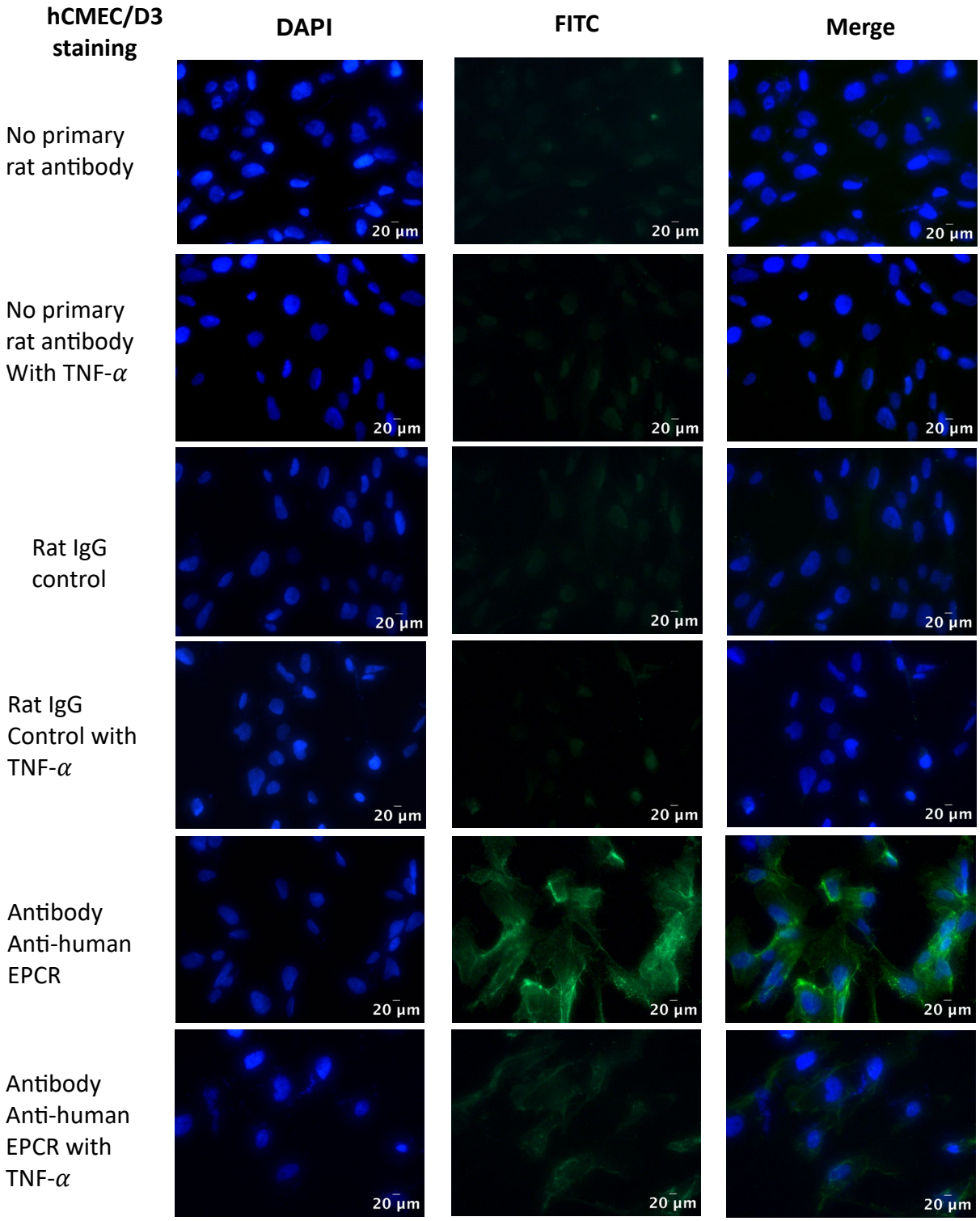


Figure 3-5:Characterisation of hCMEC/D3 with rat monoclonal antibody to human EPCR. In resting and activated conditions, cells were fixed with 1% paraformaldehyde, blocked with 5% goat serum then stained with primary antibody according to manufacturer's instructions.

Immunofluorescence was detected by an appropriate secondary antibody Alexa fluor 488 conjugated and read with FITC filter (green) and counterstained the cell nuclei with DAPI and read with DAPI filter (Blue). Independent experiments were conducted at least twice for each candidate receptor and images shown are representative. All pictures were taken at x400 with a scale bar of 20mm.

Most of these results are in line with previous findings, as summarised in table below (Table 3-1). However, some differences exist, C1QBP was previously reported to be surface-expressed (Biswas et al., 2007). Resting hCMEC/D3 staining with anti-human VCAM1 was previously reported to be negative (Weksler et al., 2005). Resting and activated HBEC stained with anti-human TSP was previously described as positive (Xiao et al., 1996), CSA and integrin αvβ3 were also found positive (Wassmer et al., 2006).

Table 3-1: Summary of HBEC receptors localisation on hCMEC/D3.

	Surface expression		Intracellular expression		Findings vs Reference	Reference
Known HBEC receptors	Resting hCMEC/D3	Activated hCMEC/D3	Resting hCMEC/D3	Activated hCMEC/D3		
C1QBP	-	-	+	+	Disagree	Biswas et al, 2007
ICAM1	+	+++	+	+	Agree	Weksler et al, 2005
PECAM1	++	++	++	++	Agree	Weksler et al, 2005
TSP	-	-	+	+	Disagree	Xiao et al, 1996
CD62E	-	-	-	-	Agree	Wassmer et al,2006
CD62P	-	-	-	-	Agree	Navratil et al, 1997
CD36	-	-	-	-	Agree	Weksler et al, 2005
Integrin αv	-	-	-	-	Disagree	Wassmer et al, 2006
Fractalkine	-	-	-	-	Agree	Avril et al, 2012
NCAM	-	-	-	-	Agree	Pouvelle et al, 2007
VCAM1	++	++	++	++	Disagree	Weksler et al, 2005
EPCR	+	+/-	-	+	Agree	Simmonds et al, 1999
CSA	-	-	-	-	Disagree	Wassmer et al, 2006
sC1QBP + C1QBP	+	+	+	+	Agree	Ghebrehiwet et al, 2014

NB: Receptors colored in blue are constitutively expressed on hCMEC/D3. However, receptors colored in black are not constitutively expressed by hCMEC/D3, and the receptor colored in red can become associated with hCMEC/D3 when added exogenously or from normal human plasma in the cell culture medium.

3.5.2. Soluble C1QBP provides additional cell surface receptor for hCMEC/D3

My initial finding that C1QBP was not detected on the surface of hCMEC/D3 was unexpected, given its previous identification as an endothelial cell receptor for *P. falciparum* cytoadhesion (Biswas et al., 2007). The sub-cellular localization of C1QBP has been controversial in the literature (van den Berg et al., 1997; van Leeuwen & O'Hare, 2001), so I decided to investigate this further. One previous study demonstrated the ability of exogenously added soluble C1QBP to bind to the membrane of human endothelial cells (Ghebrehiwet et al., 2014). To test whether soluble C1QBP could adhere hCMEC/D3, the cells were incubated with 2.5 μg/mL in cell culture medium for 2 hours at 37ºC before staining with antibody to human C1QBP. After this pre-incubation with soluble protein, resting and activated hCMEC/D3 cells exhibited surface membrane expression for C1QBP, in contrast to their negative controls (Figure 3-6 rows 1 to 6 from the top).

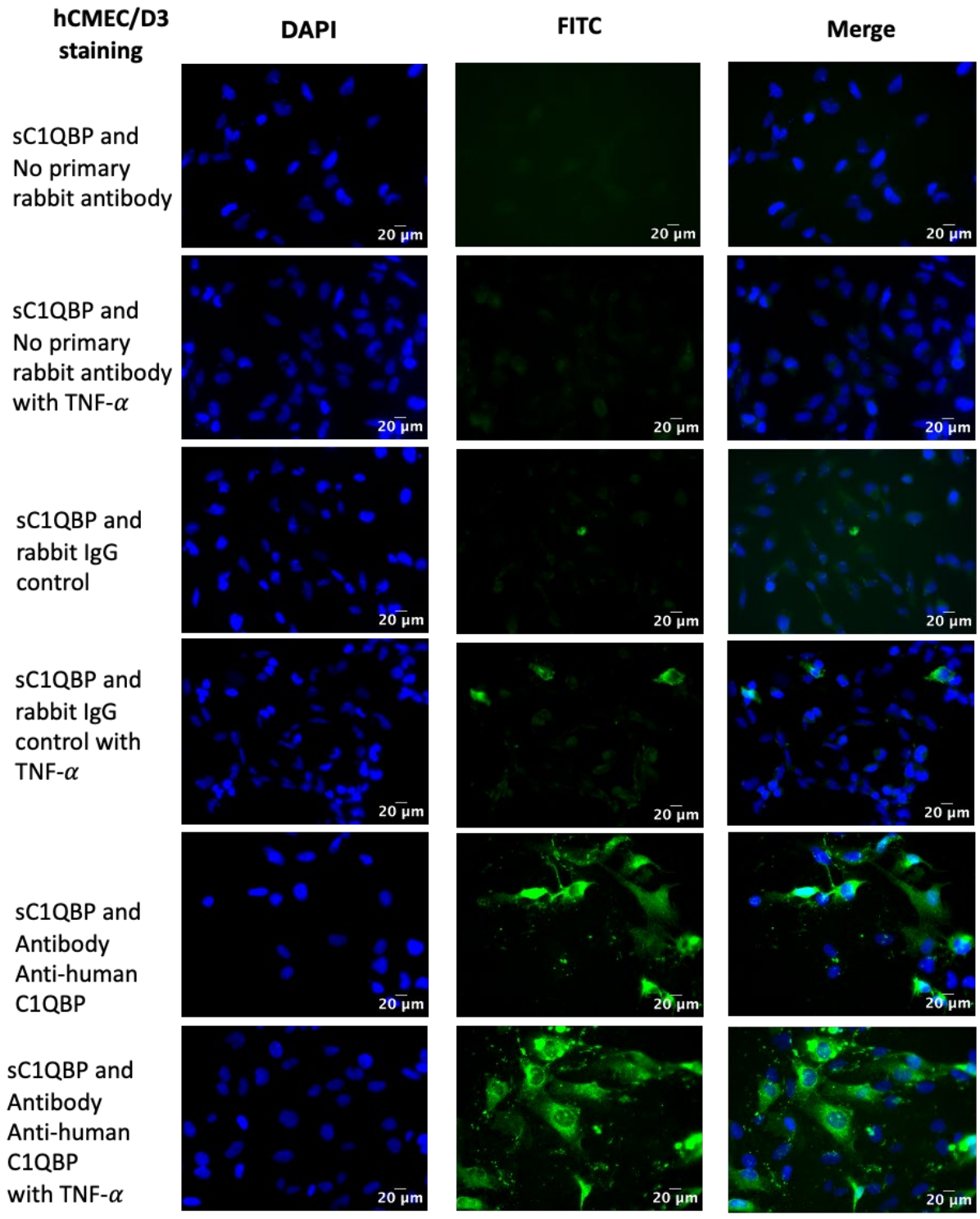


Figure 3-6. Preincubation of hCMEC/D3 with soluble C1QBP and staining with rabbit polyclonal antibody to human C1QBP. Resting and activated hCMEC/D3 cells were preincubated with soluble C1QBP, then cells were fixed with 1% paraformaldehyde, blocked with 5% goat serum, and stained with rabbit polyclonal antibody to human C1QBP. Immunofluorescence was detected by a secondary Alexa fluor 488 goat anti-rabbit IgG and read with FITC filter (green) and counterstained the cell nuclei with DAPI and read with DAPI filter (Blue). Independent experiments were conducted at least twice and images showed are representative. All pictures were taken at x400 magnification with a scale bar of 20µm.

A previous study has demonstrated the presence of soluble C1QBP in eight normal human plasmas (van den Berg et al., 1997). Therefore, I tested the physiological relevance of soluble C1QBP adhering to resting and activated hCMEC/D3. hCMEC/D3 cells were incubated with 10% normal human plasma in complete DMEM/ F-12 medium for 24 hours before IFA. hCMEC/D3 cells show expression of C1QBP on the cell surface of some resting and activated hCMEC/D3 (Figure 3-7, rows 1 to 6 from the top). Collectively, recombinant C1QBP and soluble C1QBP from normal human plasma improve the detection of surface membrane-associated C1QBP on resting and activated hCMEC/D3.

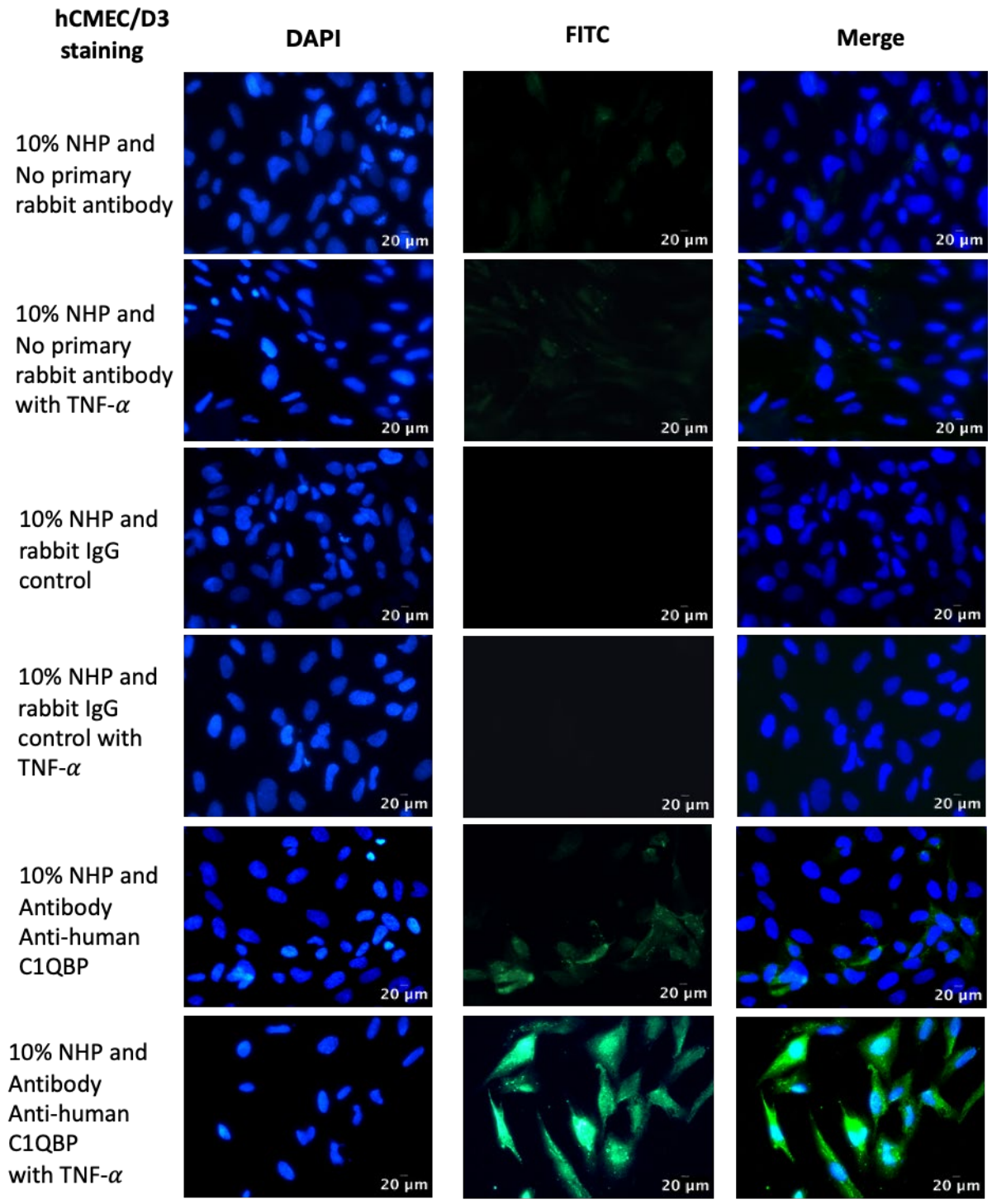


Figure 3-7. Preincubation of hCMEC/D3 with 10% normal human plasma and staining with rabbit polyclonal antibody to human C1QBP. In resting and activated conditions, cells were fixed with 1% paraformaldehyde, blocked with 5% goat serum then stained with primary antibody according to manufacturer's instructions. Immunofluorescence was detected by an appropriate secondary antibody Alexa fluor 488 conjugated and read with FITC filter (green) and counterstained the cell nuclei with DAPI and read with DAPI filter (Blue). Independent experiments were conducted at least twice and images shown are representative. All pictures were taken at x400 magnification with a scale bar of 20µm.

3.5.3. Soluble C1QBP is present in normal human plasma and during *P. falciparum* infection

The above section shows that soluble C1QBP from normal human plasma can become associated with the membrane of resting and activated hCMEC/D3 and thereby serve as a potential receptor for IE sequestration (Figure 3-6). The presence of 18-30 ng/mL of C1QBP in normal human serum was noted in one previous study (van den Berg et al., 1997), but other reports are lacking. Hence, I investigated whether I could confirm this finding.

A commercial sandwich ELISA kit was used to detect and quantify the presence of soluble C1QBP in two plasma samples collected from a healthy UK adult, and eight frozen plasma samples from African children infected with *P. falciparum*. I prepared a series of dilutions of a known concentration of recombinant C1QBP (standard) (section 2.4.4), and samples of unknown concentration (section 2.4.3). Then, I measure their absorbance at 450 nm with an ELISA plate reader, I plotted the data in GraphPad Prism version 9.5.1, and I generated a standard curve (Figure 3-8).

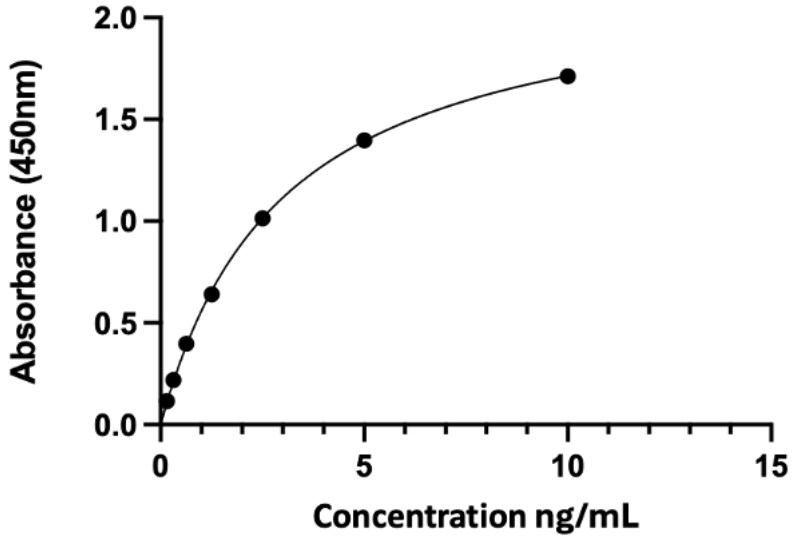


Figure 3-8. Standard curve of absorbance (450 nm) against concentration of recombinant C1QBP (ng/mL). Using recombinant C1QBP as a standard, an ELISA test with a sensitivity of 10 ng/mL was performed. On the x-axis the concentration of the sample can be read, and on the y-axis the absorbance value. Each black dot on the sigmoidal curve represents a known dilution concentration of the standard.

Since plasmas from African children were cryopreserved for a long period, I decided to assess whether the long-term storage did not degrade the protein C1QBP. I collected fresh plasma from a malaria naive UK-based adult, cryopreserved them at 4°C and -70°C for more than three

weeks (considered as long-term storage). The day of the assay, I collected a new plasma from the same healthy adult and tested with his previous plasma collected and stored for at least 3 weeks. The concentration of C1QBP in freshly collected adult plasma is 12.4 ng/mL (Table 3-2), and plasmas stored for three weeks at 4°C and at -70°C are 16.0 ng/mL and 10.5 ng/mL respectively. Therefore, C1QBP levels are equivalent in freshly collected and cryopreserved plasma over this timescale (4°C and -70°C for at least 3 weeks). However, I notice that the plasma concentration of fresh samples diluted at 1/2 and 1/4 is over the limit detection of the ELISA kit.

Table 3-2. C1QBP levels in UK adult plasma -collected freshly and cryopreserved for at least three weeks

Sample	Concentration adjusted by dilution factor (ng/mL)	Concentration Median
Sample (-70ºC) 1/2	ND	10.5
Sample (-70ºC) 1/4	10.5	
Sample (4ºC) 1/2	24.1	16.0
Sample (4ºC) 1/4	8	
fresh sample undiluted	12.4	12.4

ND: non determined

The median C1QBP level in plasma samples from eight children suffering from malaria is 1.6 ng/mL (Table 3-3). Seven samples had values <3 ng/mL, while one had a plasma C1QBP concentration of 27.4 ng/mL (Table 3-3). Since malaria patients can be classified based on their clinical disease syndromes (severe versus uncomplicated malaria), the level of soluble C1QBP in plasma of children with uncomplicated (n=4) versus severe malaria (n=4). I do not observe any difference in plasma C1QBP level between children suffering from uncomplicated versus severe malaria in this small dataset (Table 3-3). Overall, these results suggest that C1QBP is present in naïve malaria normal human plasma and in the plasma of children affected by malaria.

Table 3-3. Plasma of children infected with *P. falciparum* reactivity to recombinant C1QBP**.**

ID SM cases	Conc adjusted by dilution factor	Conc median	ID UM cases	Conc adjusted by dilution factor	Conc median
AH006 1/2	1.7	1.7	**AH025 1/2**	1.1	1.5
AH006 1/4	2.7		**AH025 1/4**	1.6	
AH009 1/2	1.1		**AH027 1/2**	1.1	
AH009 1/4	1.7		**AH027 1/4**	1.5	
AH011 1/2	1.3		**AH030 1/2**	ND	
AH011 1/4	2.3		**AH030 1/4**	27.5	
AH012 1/2	1.1		**AH032 1/2**	1.1	
AH012 1/4	1.7		**AH032 1/4**	1.7	

ND: non determined **Conc:** Concentration

SM: Severe malaria **UM:** Uncomplicated malaria.

3.5.4. Immortalised HBEC-5i do not constitutively express C1QBP on their surface membrane

I next investigated whether other immortalised endothelial cell lines (HBEC-5i) exhibited similar properties as hCMEC/D3 (i.e. express C1QBP only when added exogenously or from normal human plasma). Prior to characterising the cellular localisation of C1QBP, I assessed the endothelial nature of the cell line using vWF as the endothelial cell marker, and ICAM1 as a positive control. Later, I determined whether adding soluble recombinant C1QBP improved the detection of C1QBP on this human endothelial cell line. The presence or absence of

immunofluorescent staining was determined by microscopy with comparison to appropriate negative controls (rabbit polyclonal and mouse monoclonal antibodies)

I studied the immortalised HBEC-5i line, which has been widely used in CM research (Avril et al., 2016; Azasi et al., 2018; Claessens et al., 2012; Wassmer et al., 2006). To authenticate the endothelial nature of HBEC-5i, the cells were fixed, permeabilised and labelled with rabbit polyclonal antibody to human vWF. As expected, resting HBEC-5i cells exhibit the presence of positive intracytoplasmic granule staining in contrast to their negative controls, which show only background staining (Figure 3-9, from the top rows 1, 2, and 3). These results validate the endothelial origin of HBEC-5i.

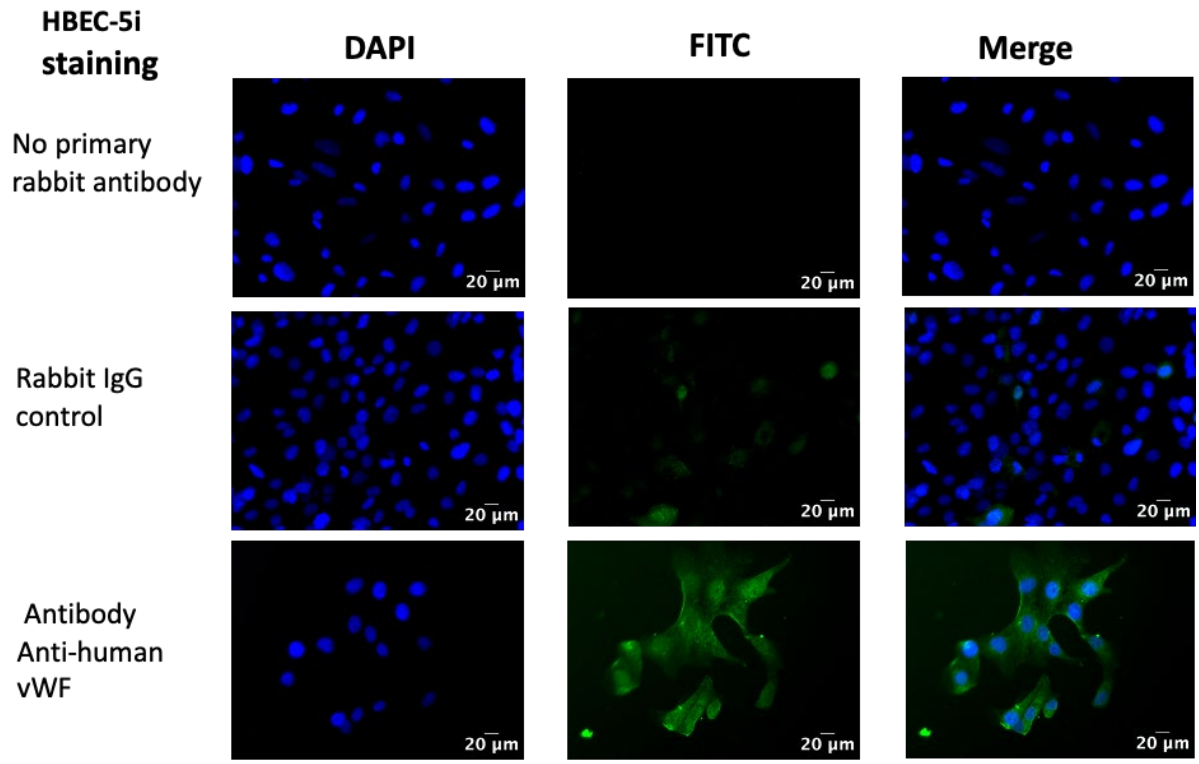


Figure 3-9. Authentication of HBEC-5i as a human endothelial cell line. Resting HBEC-5i cells were fixed with 1% paraformaldehyde, permeabilised with 0.1% Triton X-100, blocked with 5% goat serum and then stained with rabbit polyclonal antibody to human vWF. Immunofluorescence was detected using an Alexa fluor 488 goat anti-rabbit IgG and visualised with FITC filter (green), counterstained nuclei with DAPI and visualised with the corresponding filter (Blue). Independent experiments were conducted at least twice for this marker and images shown are representative. All pictures were taken at x400 magnification with a scale bar of 20µm.

I also fixed and stained resting and activated HBEC-5i cells with a monoclonal antibody to human ICAM1, as a positive control for human endothelial cell surface staining. Resting and activated HBEC-5i cells do display surface expression of ICAM1 (Figure 3-10, rows 1 and 2

from the bottom), with brighter staining being seen after TNF-α stimulation (Figure 3-10, row 1 from the bottom). Therefore, ICAM1 is constitutively expressed on the surface of HBEC-5i and upregulated after activation of the cells, as expected (Wassmer et al., 2006; Xiao et al., 1996; Zougbede et al., 2011).

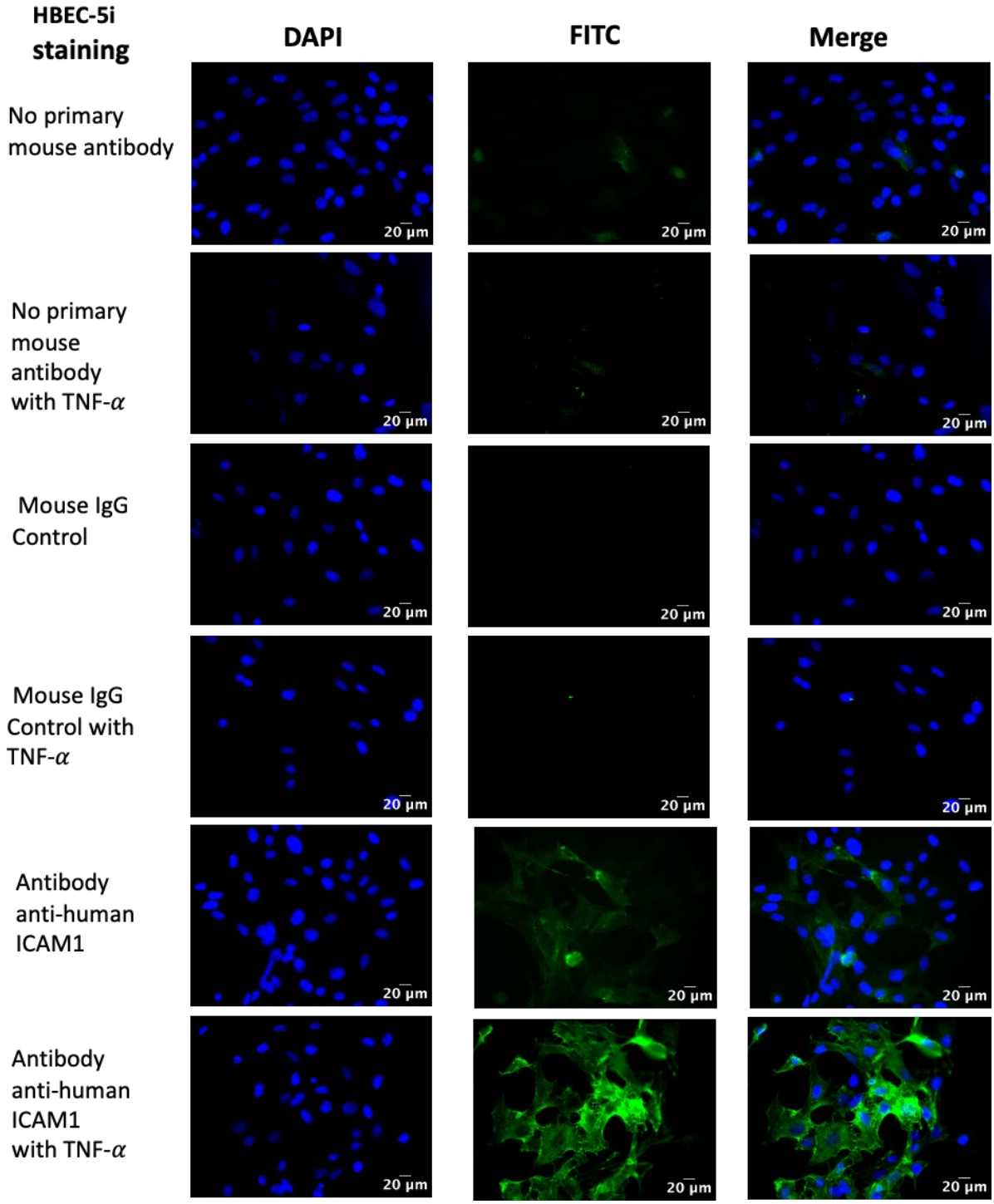


Figure 3-10. Staining of HBEC-5i cells with mouse monoclonal antibody to human ICAM1. Resting and activated HBEC-5i cells were fixed with 1% paraformaldehyde, blocked with 5% goat serum and then stained with mouse monoclonal antibody to human ICAM1. Immunofluorescence was detected using an Alexa fluor 488 goat anti-mouse IgG and visualised with FITC filter (green), counterstained nuclei with DAPI and visualised with the corresponding filter (Blue). Independent experiments were conducted at least twice for this receptor and images shown are representative. All pictures were taken at x400 magnification with a scale bar of 20μm.

Further, I examined the expression of C1QBP by HBEC-5i. Resting and activated HBEC-5i cells do not exhibit surface expression of C1QBP but show some intracellular staining distinct from their negative controls, which display only background level staining (Figure 3-11, rows 1 and 2 from the bottom and rows 1, 2, 3 and 4 from the top). Therefore, C1QBP is not constitutively exhibited on the surface of HBEC-5i.

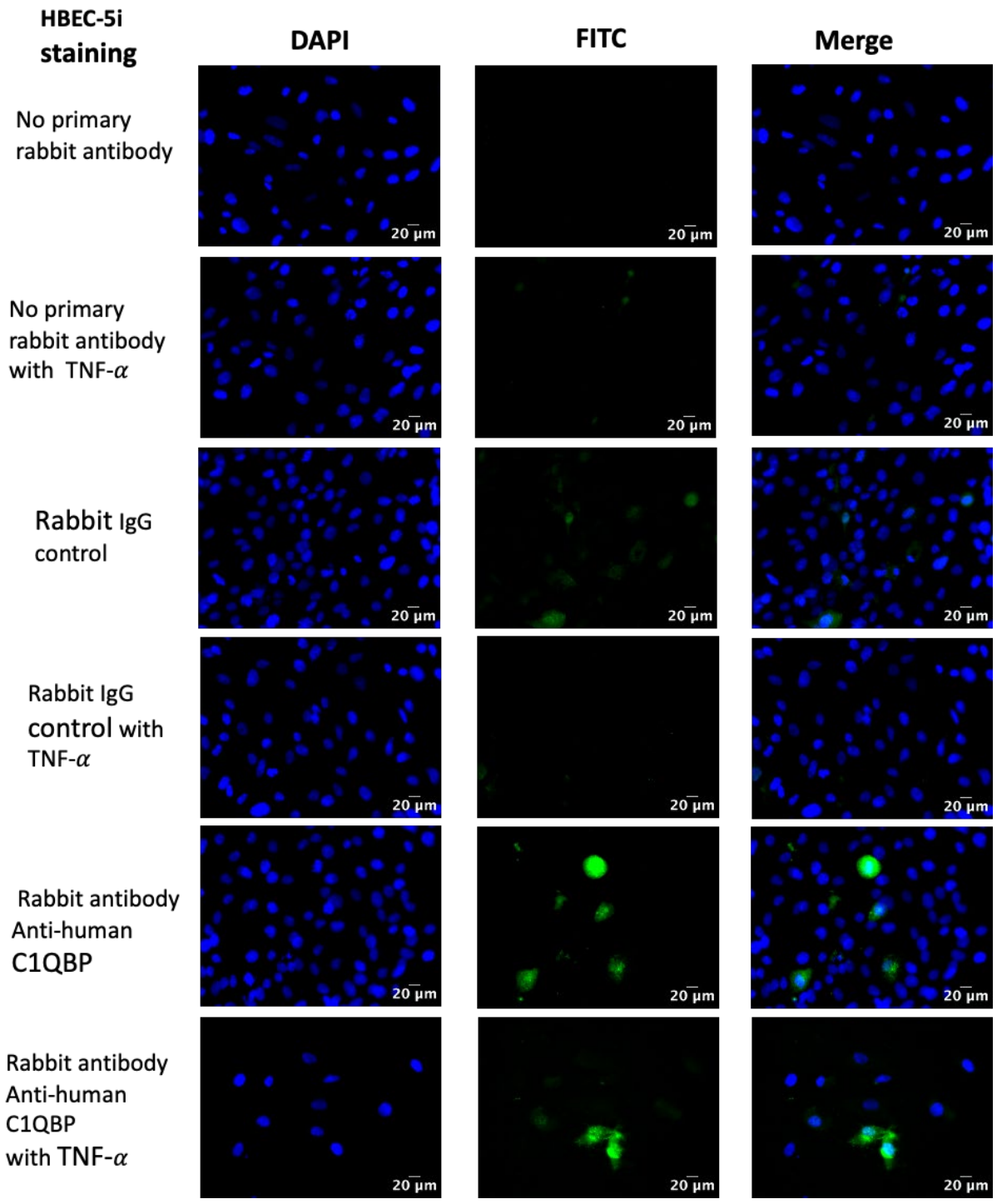


Figure 3-11. Staining of HBEC-5i with rabbit polyclonal antibody to human C1QBP. Resting and activated HBEC-5i cells were fixed with 1% paraformaldehyde, blocked with 5% goat serum and then stained with rabbit polyclonal antibody to human C1QBP. Immunofluorescence was detected using an Alexa fluor 488 goat anti-rabbit IgG and visualised with FITC filter (green), counterstained nuclei with DAPI and visualised with the corresponding filter (Blue). Independent experiments were conducted at least twice for this receptor and images shown are representative. All pictures were taken at x400 magnification with a scale bar of 20µm.

As the immortalised HBEC-5i line showed a similar staining pattern to hCMEC/D3 (Figure 3-11, rows 1 and 2 from the bottom; Figure 3-3, rows 1 to 6 from the top respectively), I decided to investigate whether soluble C1QBP could bind to HBEC-5i. I preincubated HBEC-5i with recombinant C1QBP before staining with rabbit polyclonal antibody to human C1QBP. Although much of the staining was over the cell nuclei, there was also staining consistent with C1QBP surface expression of, especially under activated conditions (Figure 3-12, rows 1 and 2 from the bottom). Therefore, HBEC-5i shows results like hCMEC/D3, suggesting the possibility of soluble C1QBP acting as a bridging molecule to mediate the cytoadhesion of IE to HBECs.

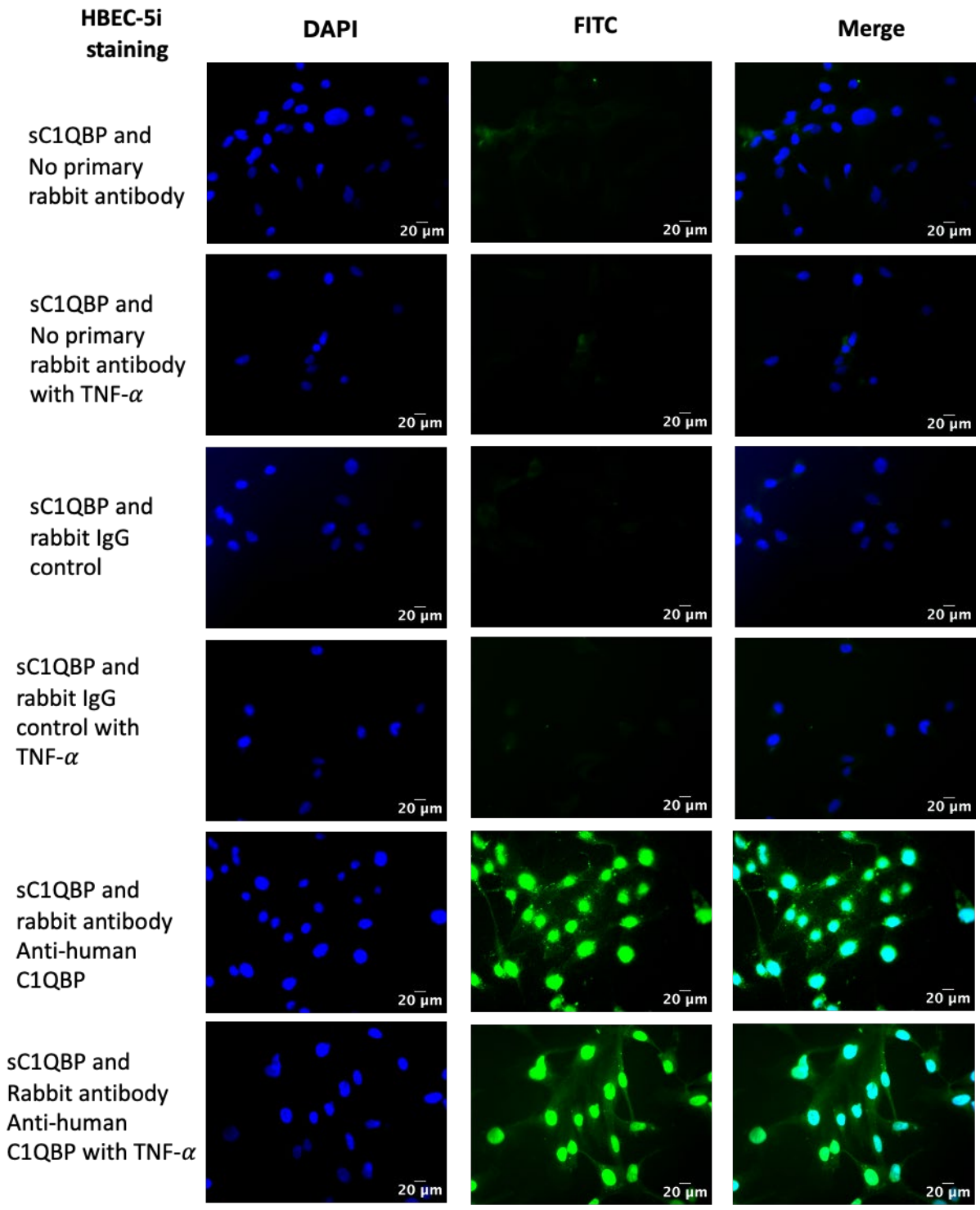


Figure 3-12. Preincubation of HBEC-5i with soluble C1QBP and staining with rabbit polyclonal antibody to human C1QBP. Resting and activated hCMEC/D3 cells were preincubated with soluble C1QBP,, cells were fixed with 1% paraformaldehyde, blocked with 5% goat serum, and stained with rabbit polyclonal antibody to human C1QBP. Immunofluorescence was detected by a secondary Alexa fluor 488 goat anti-rabbit IgG conjugated and read with FITC filter (green) and counterstained the cell nuclei with DAPI and read with DAPI filter (Blue). Independent experiments were conducted at least twice and images shown are representative. All pictures were taken at x400 magnification with a scale bar of 20mm.

3.5.5. Primary endothelial cells constitutively express C1QBP on their surface membrane

To confrim the endothelial origin of primary HBMEC, the cells were fixed, permeabilised and stained with rabbit polyclonal antibody to human vWF. As expected, resting primary HBMEC cells show the presence of positive intracytoplasmic granule staining distinctly to their negative controls, which exhibit only background staining (Figure 3-13, rows 1, 2, and 3 from the top). These results confirm the endothelial origin of primary HBMEC.

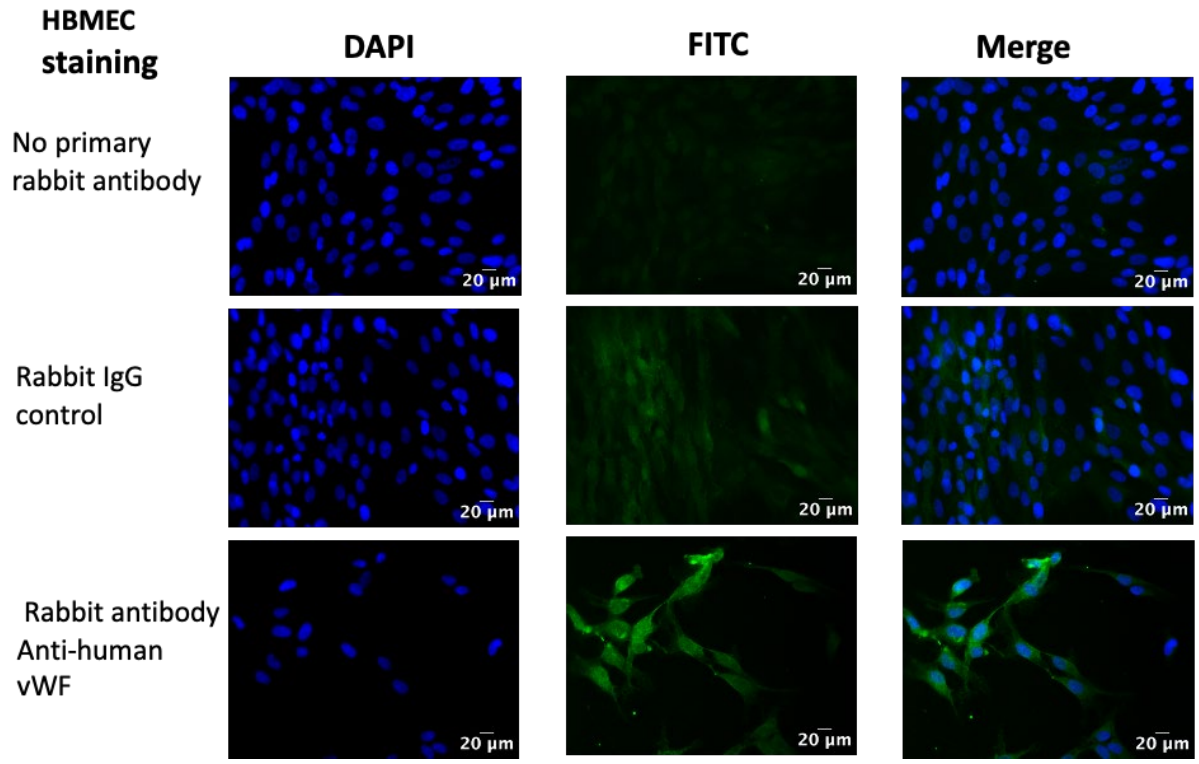


Figure 3-13. Authentication of primary HBMEC as a human endothelial cell line. Resting primary HBMEC cells were fixed with 1% paraformaldehyde, permeabilised with 0.1% Triton X-100, blocked with 5% goat serum and then stained with rabbit polyclonal antibody to -human vWF. Immunofluorescence was detected by an Alexa fluor 488 goat anti-rabbit IgG and visualised with FITC filter (green), counterstained nuclei with DAPI and visualised with the corresponding filter (Blue). Independent experiments were conducted at least twice for this marker and images shown are representative. All pictures were taken at x400 magnification with a scale bar of 20µm.

I also labelled fixed primary HBMEC in resting and activated conditions with a monoclonal antibody to human ICAM1 as a positive control for human endothelial cells. Resting and activated primary HBMEC do show surface expression of ICAM1, which is more marked after

TNF-α stimulated cells (Figure 3-14, rows 1 and 2 from the bottom). Therefore, ICAM1 is constitutively displayed on the surface of primary HBMEC in resting and activated conditions.

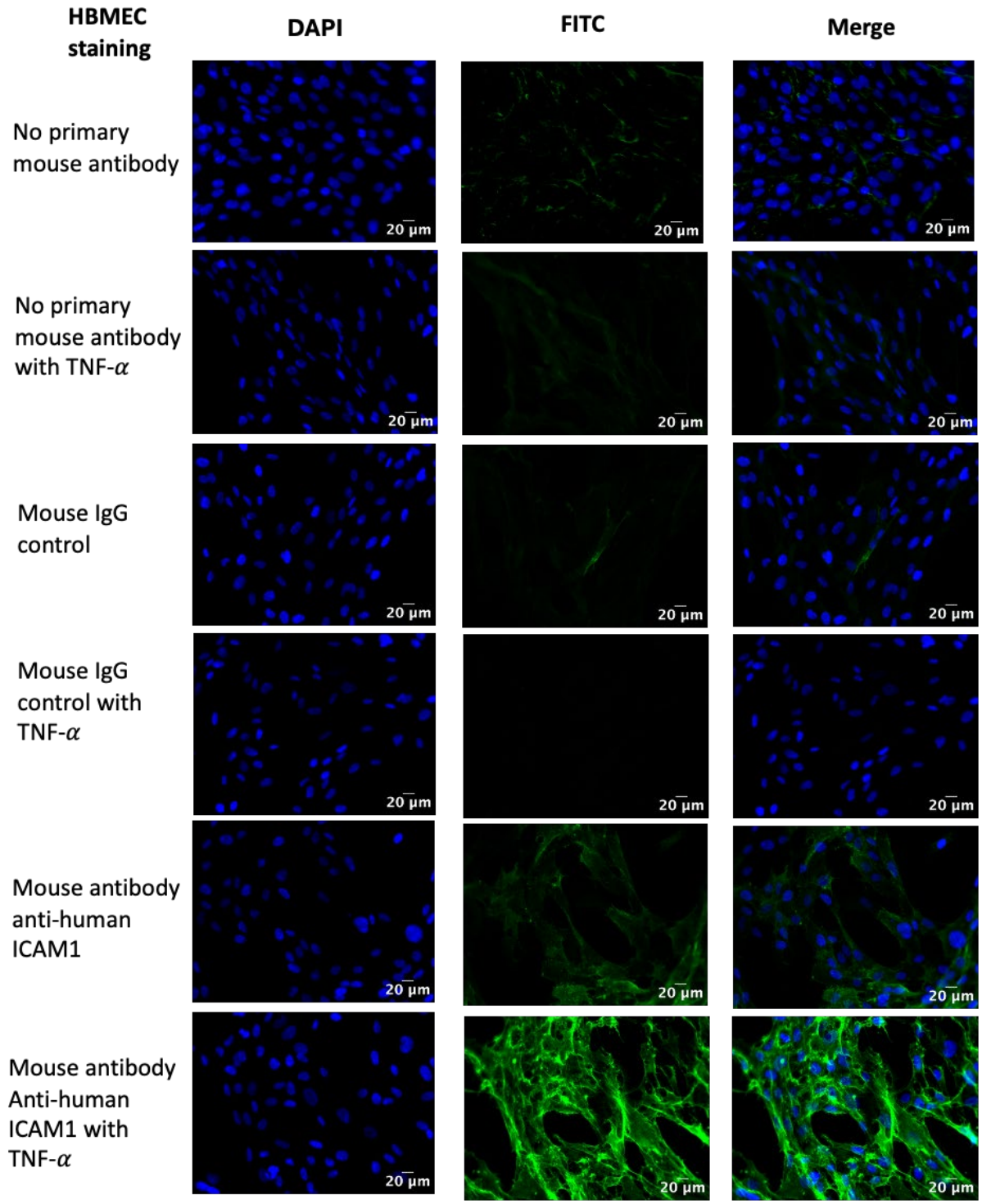


Figure 3-14. Staining of primary HBMEC cells with a mouse monoclonal antibody to human ICAM1. Resting and activated primary HBMEC cells were fixed with 1% paraformaldehyde, blocked with 5% goat serum and then stained with a mouse monoclonal antibody to human ICAM1. Immunofluorescence was detected by an

Alexa fluor 488 goat anti-mouse IgG and visualised with FITC filter (green), counterstained nuclei with DAPI and visualised with the corresponding filter (Blue). Independent experiments were conducted at least twice for this receptor and images shown are representative. All pictures were taken at x400 magnification with a scale bar of 20μm.

To assess whether C1QBP is constitutively expressed on primary endothelial cells, I examined the expression of C1QBP by primary HBMEC. Fixed primary HBMEC cells in resting and activated conditions constitutively exhibit bright staining consistent with surface expression of C1QBP, distinct from their negative controls, which display background level staining (Figure 3-15, rows 1 and 2 from the bottom and rows 1, 2, 3 and 4 from the top). Therefore, unlike the results from the immortalised HBEC lines, C1QBP is constitutively express by primary HBMEC in resting and TNF-α activated conditions.

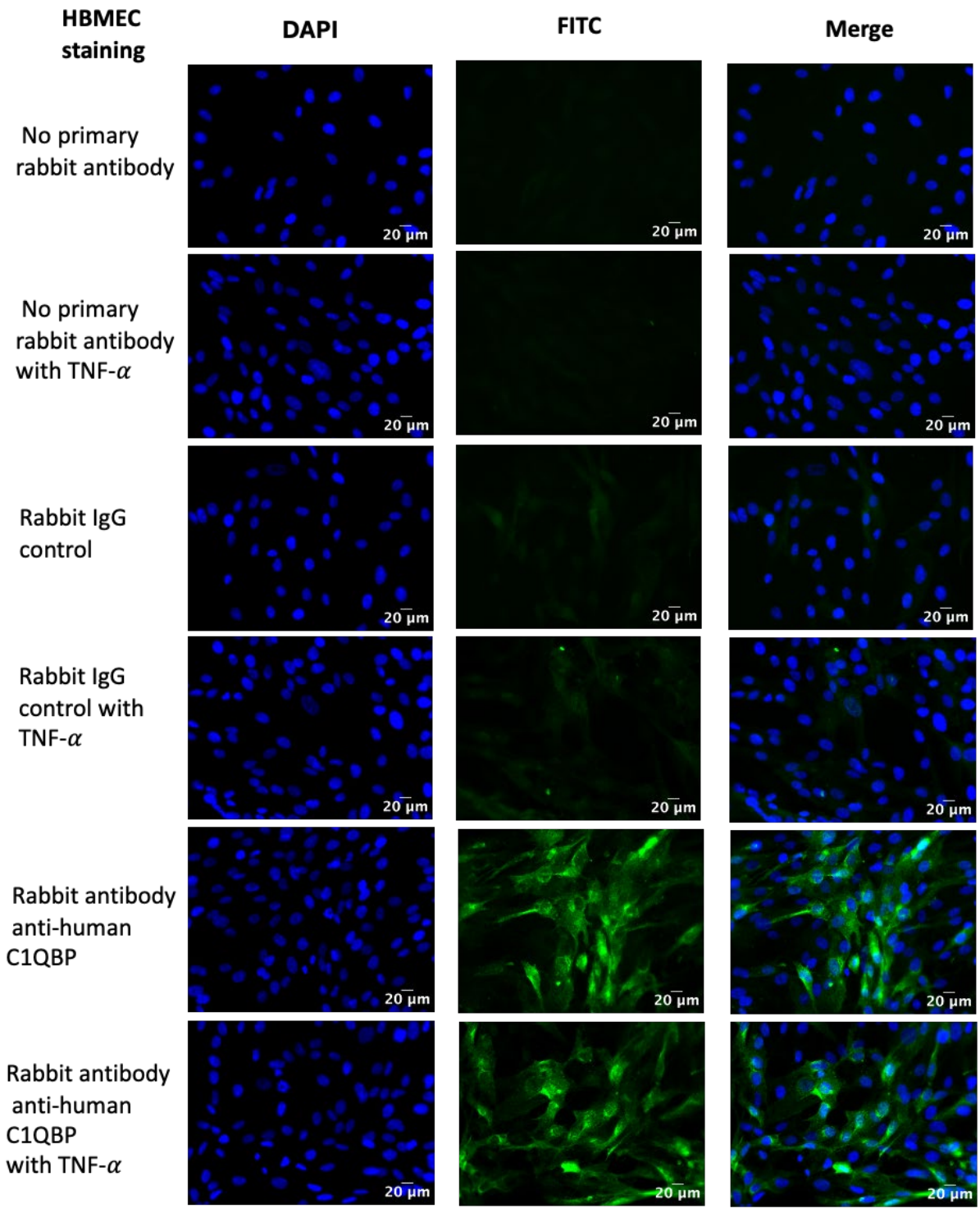


Figure 3-15. Staining of primary HBMEC with rabbit polyclonal antibody to human C1QBP. Resting and activated primary HBMEC cells were fixed with 1% paraformaldehyde, blocked with 5% goat serum and then stained with rabbit polyclonal antibody anti-human C1QBP. Immunofluorescence was detected by an Alexa fluor 488 goat anti-rabbit IgG and visualised with FITC filter (green), counterstained nuclei with DAPI and visualised with the corresponding filter (Blue). Independent experiments were conducted at least twice for this receptor and images shown are representative. All pictures were taken at x400 magnification with a scale bar of 20µm.

The preincubation of the immortalised lines hCMEC/D3 and HBEC-5i with recombinant C1QBP before staining with rabbit polyclonal antibody to human C1QBP enhanced the surface detection of C1QBP in IFA (Figure 3-6 and Figure 3-12). Therefore, I explored if the same applied to primary HBMEC. Very bright staining is seen after pre-incubating with soluble CIQBP, but further experiments using a quantitative technique would be needed to determine if the staining is increased.

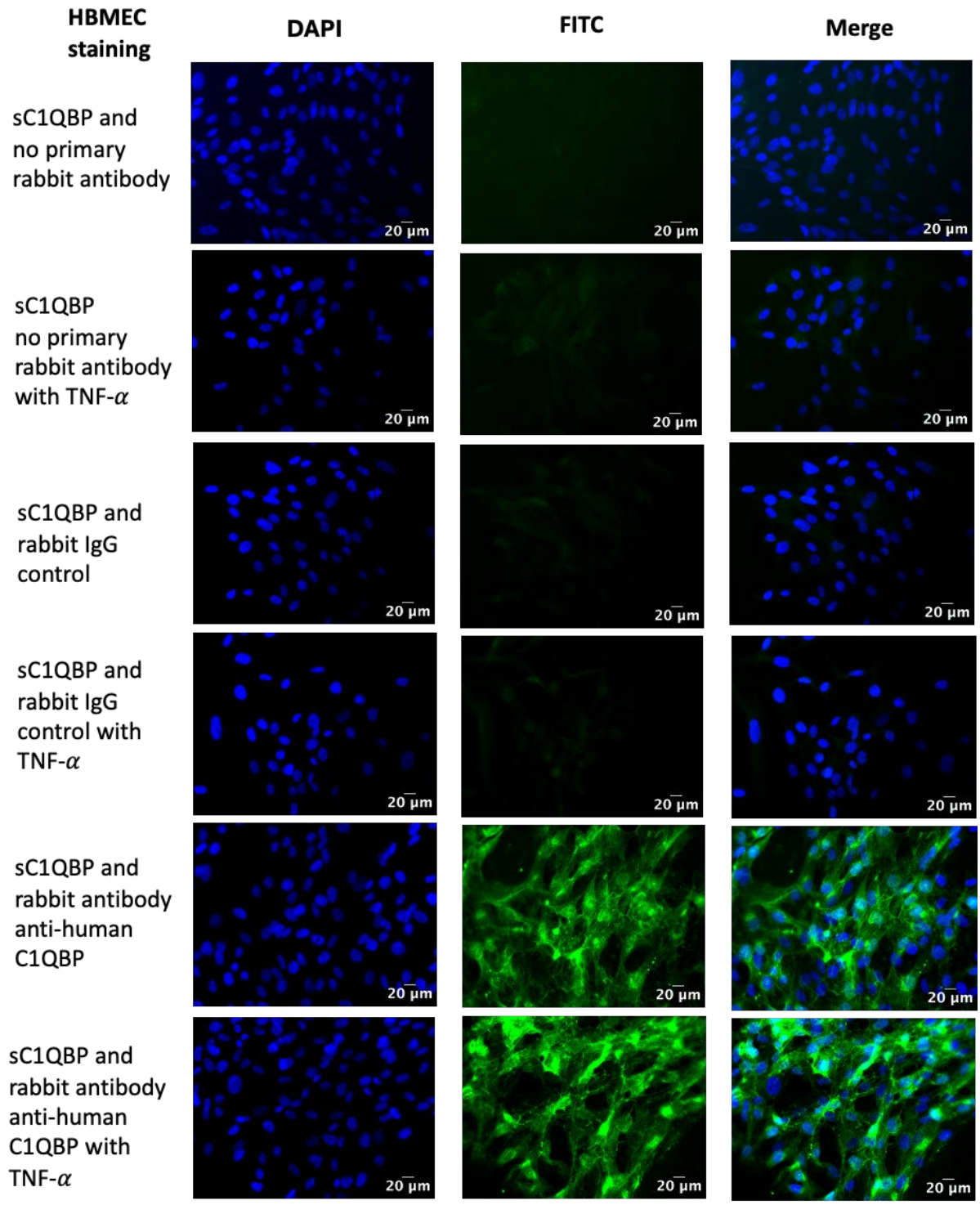


Figure 3-16. Preincubation of primary HBMEC with soluble C1QBP and staining with rabbit polyclonal antibody to human C1QBP. Resting and activated primary HBMEC cells were preincubated with soluble C1QBP, cells were fixed with 1% paraformaldehyde, blocked with 5% goat serum, and stained with rabbit polyclonal antibody to human C1QBP. Immunofluorescence was detected by a secondary Alexa fluor 488 goat anti-rabbit IgG conjugated and read with FITC filter (green) and counterstained the cell nuclei with DAPI and read with DAPI filter (Blue). Independent experiments were conducted at least twice and images shown are representative. All pictures were taken at x400 magnification with a scale bar of 20µm.

The same experiments were repeated with two more primary human endothelial cell lines, HPMEC and HDMEC. The data are shown in appendix (Figure 6-1, 6-2, 6-3, 6-4, 6-5, 6-6, and 6-7) and summarised below in Table 3-4 . Both cell lines showed the same results as HBMEC, with bright staining consistent with surface expression of C1QBP in both resting and activated cells.

Table 3-4: Summary of C1QBP localisation on human endothelial cells.

Cell line	Organ/ Source	Immortalised/ Primary	Constitutive Surface C1QBP	Exogenous Surface C1QBP
hCMEC/D3	Brain/ Sigma cat # SCC066	Immortalised	No	Yes
HBEC-5i	Brain/ ATCC cat # crl-3245	Immortalised	No	Yes
HBMEC	Brain Sciencell cat # 1000	Primary	Yes	Yes
HPMEC	Lung Sciencell cat # 3000	Primary	Yes	Yes
HDMEC	Skin Sciencell Cat # 2000	Primary	Yes	Yes

3.6. Discussion

3.6.1. Summary of major findings

The main objective of this chapter was successfully achieved: hCMEC/D3, HBEC-5i, primary HBMEC, HPMEC, and HDMEC were stained with rabbit polyclonal antibody to human C1QBP to determine C1QBP cellular localisation using IFA and fluorescence microscope. There was a striking difference in the surface membrane expression of C1QBP between immortalised human endothelial cells (hCMEC/D3 and HBEC-5i) and primary human endothelial cells (HBMEC, HPMEC, and HDMEC). Primary human endothelial cells constitutively showed consistent surface membrane expression of C1QBP, but immortalised human endothelial cells did not. However immortalised human endothelial cells can exhibit surface expression of C1QBP after preincubation of endothelial cells with recombinant C1QBP or human plasma. These findings indicate that immortalised human brain endothelial cells may not retain all the biological characteristics of primary human brain endothelial cells like the surface expression of C1QBP in this study.

3.6.2. Validation of hCMEC/D3 and other human endothelial cells

The authentication of the endothelial nature of immortalised (hCMEC/D3 and HBEC-5i) and primary (HBMEC, HPMEC, and HDMEC) human endothelial cells is important because human endothelial cell receptors play a role in the occurrence of CM (Turner et al, 2013; Biswas et al, 2007; Berendt et al,1989; Barnwell et al,1995). I investigated hCMEC/D3 and other endothelial cells (HBEC-5i, primary HBMEC, HPMEC, and HDMEC) for their ability to exhibit specific human endothelial cells markers. These markers are vWF, CD31/PECAM1, Dil-Ac-LDL, and SMA. SMA is a specific marker for fibroblast, which is the first contaminant for primary human brain endothelial cells. SMA was used as a negative control.

As expected hCMEC/D3 showed positive staining to vWF, PECAM1/CD31, Dil-Ac-LDL, and negative staining for SMA. The other endothelial cells have been validated using vWF, the specific marker for human endothelial cells and ICAM1 as a positive control. In all experiments the absence of primary antibody staining and the staining with an irrelevant IgG were also used as negative control. All endothelial cells have displayed positive staining to vWF and ICAM1. These results confirm the endothelial nature of these cell lines and are in line with previous

work (Azasi et al., 2018; Wassmer et al., 2006; Weksler et al., 2005). These results also suggest the use of these endothelial cell lines as a model for investigating CM.

3.6.3. Lack of C1QBP surface expression on immortalised hCMEC/D3

A few prior studies have used the immortalised human brain microvascular endothelial cell line hCMEC/D3 to understand CM pathogenesis (Jambou et al., 2010; Zougbede et al., 2011). In my study, fixed hCMEC/D3 cells were used in IFAs to characterise C1QBP and twelve known receptors involved in *P. falciparum* cytoadherence. Most of the results were as expected, with staining for key receptors such as ICAM1 and EPCR being detected (Table 3-1, discussed further below). However, one notable exception was that hCMEC/D3 cells showed intracellular staining but no surface membrane expression for C1QBP in either resting or activated conditions (Figure 3-2, rows 5 and 6 from the top).

This result was unexpected because Biswas et al. reported surface detection of C1QBP on HUVEC, immortalised HBEC, and primary HBMEC before and after stimulation with TNF-α by flow cytometry (Biswas et al., 2007). Closer examination of the data in Biswas et al., showed that the evidence for surface expression was based on flow cytometry MFIs (median fluorescence intensities), but no images to support the conclusion of surface staining were provided. In both studies, Biswas et al. and experiments described here, HBEC cells were fixed but not permeabilized, so that theoretically, only surface expression rather than internal staining should be obtained. However, as shown here (Figure 3-2, rows 5 and 6 from the top), antibodies are often able to access the interior of the cell after fixation. Hence, the positive MFIs described by Biswas et al. could be due to internal instead of surface expression. It also remains possible that there is another reason to explain the lack of surface expression of C1QBP found here. For example, Biswas et al, used a different antibody (generated in house), therefore my results cannot directly be compared to theirs. I used a commercial rabbit polyclonal antibody from Abcam.

3.6.4. Controversy of C1QBP localisation in the literature

The cellular localisation of C1QBP has proved controversial over the past three decades. Immunofluorescence studies have reported the surface detection of C1QBP for B cells, neutrophils, platelets, and endothelial cells (Daha et al., 1988; Eggleton et al., 1995; Ghebrehiwet et al., 1994; Peerschke & Ghebrehiwet, 1987). However, other studies have

demonstrated that C1QBP is in the cytoplasm of B cells, and in the vesicular fraction of endothelial cells (Dedio et al., 1998; van den Berg et al., 1997).

Although C1QBP (also known as gC1qR) was initially described as a cell surface receptor for the globular heads of the complement component C1q (Ghebrehiwet et al., 1994), it was independently described as a mitochondrial protein P32 and thought to be involved in mitochondrial oxidative phosphorylation (Muta et al., 1997). Many studies show a primarily mitochondrial sub-cellular localization for C1QBP, and it has been suggested that the highly acidic nature of the protein could cause artefactual binding to ligands and cell surfaces due to non-specific electrostatic effects (van Leeuwen & O'Hare, 2001). However, it has also been argued that soluble C1QBP can bind to the surface of HBEC and provide additional receptor sites for pathogens (Ghebrehiwet et al., 2014). More recently, human endothelial surface membrane C1QBP has been reported to interact with high-molecular weight kininogen and vitronectin to mediate *Candida spp* infection (Phan et al., 2022).

Given the lack of detectable C1QBP on hCMEC/D3 cells and the above evidence, I decided to assess whether soluble C1QBP can adhere to the surface of HBEC and improve host C1QBP surface membrane expression. Some resting and activated hCMEC/D3 cells did exhibit surface expression of C1QBP after preincubation with soluble C1QBP or plasma (Figure 3-5, rows 1 and 2 from the bottom). These results confirm the observation of the previous study (Ghebrehiwet et al., 2014).

3.6.5. C1QBP is present in human plasma

A previous study found a range of 18-30 ng/mL of soluble C1QBP in eight different sera from healthy humans (van den Berg et al., 1997). My data showing that C1QBP becomes associated with HBEC membranes after incubation with human plasma suggests that C1QBP is also present in plasma. To confirm this and to quantify the amount of soluble C1QBP in plasma I used a commercial ELISA kit. I found that the concentration of soluble C1QBP in the normal adult plasma sample was 12.5 ng/mL (Table 3-1). These results are in line with the ability of plasma/serum protein such as fibrinogen, high-molecular weight kininogen, and vitronectin to act as bridging molecules and mediate the adherence of pathogens to human endothelial cells (Cheung et al., 1991; Phan et al., 2022).

3.6.6. Pilot study on C1QBP in plasma from malaria patients

I had access to some cryopreserved plasma samples of African children experiencing uncomplicated or severe malaria, so I decided to investigate plasma C1QBP levels in a small subset as a pilot study. The samples have been long-term cryopreserved (-70ºC for 7 years). Therefore, I also tried to examine whether cryopreservation affected C1QBP level but could only do this over a short period. I compared the concentration of soluble C1QBP in normal adult plasma freshly collected and cryopreserved at 4ºC and -70ºC for at least three weeks from the same adult. The storage of plasma (three weeks) at 4ºC and -70ºC before the analysis did not change the result when compared to the plasma freshly collected from the same adult (Table 3-1). However, the plasma concentrations of soluble C1QBP for 7/8 patients infected with *P. falciparum* were lower than the normal adult value (Table 3-1 and Table 3-2).

These differences in soluble C1QBP concentration might be due to the long-term storage of the African samples or could be explained by age, the presence of *P. falciparum*, or the place of residence. It will be relevant to determine the plasma concentration of C1QBP in healthy children from the same age and area as malaria cases. I did not observe any marked differential expression of soluble C1QBP between African children experiencing uncomplicated and severe malaria, but a larger study would be needed to investigate this thoroughly.

3.6.7. Putative *P. falciparum* cytoadhesion receptors detected on hCMEC/D3

The hCMEC/D3 line has only rarely been used for cerebal malaria research (Jambou et al., 2010; Zougbede et al., 2011), despite being widely used to investigate other neurological conditions and BBB function (Qi et al., 2023; Weksler et al., 2013; Weksler et al., 2005). Most *in vitro* studies on cytoadhesion in CM use the HBEC-5i cell line, but it is not ideal because it lacks PECAM1/CD31(Wassmer et al., 2006), a putative cytoadhesion receptor (Treutiger et al., 1997). In this chapter, as well as investigating the cellular localisation of C1QBP on hCMEC/D3, I also explored other potential candidate receptors for *P. falciparum* cytoadhesion. hCMEC/D3 constitutively expressed ICAM1, PECAM1/CD31, VCAM1, and EPCR (Figure 3-2, 3-3 and 3-4; Table 3-1). Whereas the expression of only ICAM1 on hCMEC/D3 was up regulated after 24 hours incubation with TNF-α (Figure 3-3). The expression of ICAM1, CD31, VCAM1, and EPCR by human endothelial cells are agreed with previous observations apart the resting VCAM1 surface expression (Navone et al., 2013; Simmonds & Lane, 1999; Wassmer et al., 2006; Weksler et al., 2005; Xiao et al., 1996).

The controversy in resting hCMEC/D3 staining with antibody anti-human VCAM1 between the study of Weksler et al, and the present study could be explained by the nature of the antibody clone and the manufacturer. In both experiments cells were fixed before staining, Weksler et al, used a mouse monoclonal antibody anti VCAM1, clone 51-10C9 purchased from R&D system and this study used the clone STA from Biolegend. These antibodies may have different affinity to their target epitope under resting condition. Another reason is the method performed, Weksler et al, carried out flow cytometry and MFIs without images, but here IFA with images was performed (Figure 3-3, row 9 from the top).

3.6.8. Other putative *P. f*alciparum cytoadhesion receptors are NOT detected on hCMEC/D3

Candidate receptors such as TSP, selectin E and P, CD36, integrin αvβ3, CSA, fractalkine and NCAM were not constitutively expressed by resting and activated hCMEC/D3 (Figure 3-2 and 3-3). Most of these results are in agreement with previous findings apart the lack of hCMEC/D3 staining with human anti-TSP, anti-integrin αvβ, and CSA (Hatabu et al., 2003; Navone et al., 2013; Navratil et al., 2003; Pouvelle et al., 2007; Wassmer et al., 2006; Weksler et al., 2005; Xiao et al., 1996). The difference in resting and activated hCMEC/D3 staining with anti-human TSP in the Xiao et al study, and the research conducted here might be explained by the nature of the immortalised HBEC cell line. Xiao et al used HBEC-5i (Dorovini-Zis et al., 1991) rather than hCMEC/D3 (Weksler et al., 2005) in this research. A second reason could be the state of HBEC cells and methods used to detect the adhesion molecules. Xiao et al, did not fix their HBEC and performed an ELISA to detect TSP surface expression, without providing images. However, I fixed the HBEC and performed an IFA with relevant images (Figure 3-3, rows 5 and 6 from the bottom). A third reason might be the nature of the antibody clone and the manufacturer. Xiao et al, used a clone p10 purchased from Gibco instead of a clone A 6.1 from Invitrogen used in the present study. These antibodies may exhibit different affinity to their epitope under both, resting and activated hCMEC/D3 staining conditions. Finally, because TSP is a plasma protein, it is possible that it might become associated with the HBEC surface membrane if the cells are grown in human plasma, similar to what was seen for C1QBP. However, investigating this was outside the scope of the current project, which focusses on C1QBP.

The absence of surface membrane staining of integrin αvβ3 on hCMEC/D3 was surprising, because previous study had demonstrated the presence of integrin αvβ3 on the surface of HBEC (Wassmer et al, 2006). Both studies used immortalised human endothelial cell but from different lines, I used hCMEC/D3 and Wassmer et al work with HBEC-5i. I performed IFA and fluorescence microscopy to localise integrin αvβ3 on fixed hCMEC/D3, and previous work carried out flow cytometry and MFIs. Finally, I stained hCMEC/D3 with rabbit polyclonal human antibody IgG, clone 468255 from Abcam, and Wassmer et al labelled their HBEC-5i cells with mouse monoclonal IgG1, clone AMF7 from Immunotech.

Also, the controversy in resting and activated hCMEC/D3 staining with anti-human CSA in Wassmer et al study, and the work described here could be due to the immortalised HBEC cell line. I used hCMEC/D3 and the previous work, HBEC-5i. The clone and the manufacturer of antibodies were not similar. I used mouse monoclonal antibody IgM, clone CS56 from Sigma, and Wassmer et al, work with monoclonal antibody, clone 2030 from Temecula. Both studies used fixed HBEC, IFA on slides and fluorescence microscope. Reasons mentioned above antibody affinity can explain the difference in both studies.

3.7. Conclusions

Taking together all the results from this chapter, my data suggest that hCMEC/D3 can be a suitable cell line for research on the role of C1QBP in IE cytoadhesion, but only if the cells are grown with human plasma prior to experiments, to allow C1QBP to become associated with the HBEC membrane. Ideally, any results related to C1QBP binding obtained with an immortalised HBEC line should be confirmed using primary cells that consititutively express C1QBP.

4. Chapter IV: Investigation of the role of C1QBP in *Plasmodium falciparum* infected erythrocytes binding to immortalised human brain endothelial cells (hCMEC/D3)

4.1. Abstract

Severe and CM are associated with the expression of specific PfEMP1 subtypes, while the identity of the key host receptors involved in CM sequestration remains controversial, but may include EPCR and ICAM1. Previous work suggests that other host endothelial cell receptors such as C1QBP may also play a role in IE sequestration in the brain, but this has rarely been studied. In this thesis chapter, I used a) selected parasite lines on hCMEC/D3 expressing predominantly PfEMP1 DC8 and DC13 (IT4VAR19 and HB3VAR03 respectively) as well as other parasite lines known to exhibit PfEMP1 DC8-like (PfKE08 and 9197VAR27) and b) adhesion experiments to determine the role of C1QBP in IE adhesion to the immortalised hCMEC/D3. The selection of IE on hCMEC/D3 increases the ability of selected parasites to bind to the human endothelial cell and exhibited a particular PfEMP1 variant. In addition, in static binding assays to purified receptors, IT4VAR19-expressing IE showed significant binding to C1QBP, TSP, and EPCR. Adhesion inhibition assays showed that a monoclonal antibody (mAb) to EPCR blocked the interaction between IT4VAR19-IE and hCMEC/D3, whereas a mAb and polyclonal antibody (pAb) to C1QBP did not. HB3VAR03-IE did not bind to C1QBP, nor to any other known endothelial receptors in the static receptor assays, despite showing good binding to hCMEC/D3 cells. PfKE08 and 9197VAR27-IE did not also bind to recombinant C1QBP with. IE lines tested here adhesion to HBECs were not inhibited by human mAb and pAb to C1QBP. Overall, these results do not support C1QBP being a key host receptor in CM.

4.2. Introduction

The cytoadherence of IE to HBECs enables sequestration of IEs in vital host organs such as the brain and leads to CM, the deadliest complication of severe malaria (MacPherson et al., 1985; Taylor et al., 2004). Sequestration happens due to the molecular interaction between mature parasite proteins called PfEMP1 encoded by *var* genes, and receptors on the host endothelial lining (Rowe et al., 2009). Severe and CM are associated with expression of restricted PfEMP1 subtypes including DCs 8 and 13, but the endothelial receptors for mature parasites exhibiting these ligands are being characterised (Avril et al., 2012; Azasi et al., 2018; Claessens et al., 2012; Lavstsen et al., 2012; Reyes et al., 2024; Turner et al., 2013).

A key study used recombinant PfEMP1 to identify EPCR as a major endothelial cell molecule involved in IE sequestration (Turner et al., 2013). Subsequent studies showed that some PfEMP1 variants have dual binding specificity for EPCR and ICAM1(Avril et al., 2016), which was later confirmed by structural study (Lennartz et al., 2017). However, the physiological relevance of EPCR as the major host endothelial receptor for IE has been challenged. Some parasite lines that are predicted EPCR-binders adhere effectively to HBEC even when EPCR is blocked by antibodies or knocked down by RNAi (Azasi et al., 2018). Furthermore, exposure of other parasite lines that do bind EPCR to pool naïve malaria adult serum significantly reduced the interaction between IEs and EPCR on HBEC (Azasi et al., 2018). The precise role of EPCR in adhesion to HBEC remains controversial, with results of adhesion assays differing between research groups (Azasi et al., 2018; Bernabeu & Smith, 2017; Gillrie et al., 2015; Sampath et al., 2015; Storm et al., 2019). One plausible explanation for that discrepancy is the varying use of human serum/plasma in binding assays, as the presence of complement C1s, a protease in human serum can cleave some PfEMP1 variants at interdomain conserved sites and inhibit cytoadhesion of IE to EPCR (Azasi et al., 2021). Therefore, a complete understanding of the molecules involved in cytadhesion has not been achieved, and there is a need to look beyond EPCR for other host endothelial receptors for IE sequestration.

Previous works have identified C1QBP as a potential host receptor for IE sequestration (Biswas et al., 2007; Magallon-Tejada et al., 2016; Mayor et al., 2011). Clinical isolates and a laboratory adapted parasite line (3D7) were selected on recombinant C1QBP to increase their binding affinity. In HBEC binding inhibition assays, polyclonal antibodies to human C1QBP and recombinant C1QBP significantly reduced in a dose-dependent fashion the interaction between

IEs and HBEC (Biswas et al., 2007). These results suggest that C1QBP can mediate IE cytoadhesion. A subsequent study found that IE adhesion to C1QBP was associated with clinical phenotypes of severe malaria such as SMn and multiple seizures, but the association with CM was not investigated due to the small number of cases (Mayor et al., 2011). Another study using a laboratory adapted parasite line (IT4) showed that selection of IE on immortalised HBEC-5i led to the upregulation of the DC8-PfEMP1 variant, IT4VAR19 and a modest increase in C1QBP binding in a static adhesion assay (Claessens et al., 2012). Additionally, IE clinical isolates from Mozambican children expressing DC8 PfEMP1 demonstrated high-level binding to recombinant C1QBP, and the DBLβ12 domain of the 3D7_ PDF0020c DC8-like PfEMP1 was shown by ELISA to mediated adhesion to C1QBP (Magallon-Tejada et al., 2016). Later a computational based investigation modelled has predicted a possible molecular interaction between *P. falciparum* 3D7_PDF0020c DBLβ12 domain and human C1QBP (Bakri et al., 2021).

Taken together, the above data support the hypothesis that DC8-expressing parasite lines bind to C1QBP, probably through the DBLβ12 domain. However, more data on this adhesion phenotype of IE are needed to support previous findings. Detailed study of HBEC selected IE exhibiting either PfEMP1 DC8 or DC13 adhesion to host C1QBP is missing in the existing literature too. This chapter aims to determine whether HBEC-selected IEs expressing DC8 or non-DC8 PfEMP1 variants from several *P. falciparum* culture-adapted strains use C1QBP as a cytoadhesion receptor.

4.3. Objectives of this chapter

This chapter will aim:

1. To select assorted DC8 and DC13 expressing *P. falciparum* lines on hCMEC/D3
2. To investigate whether the hCMEC/D3-selected -IEs bind to recombinant C1QBP and other putative cytoadhesion receptors
3. To assess the ability of recombinant human C1QBP and antibodies to human C1QBP to prevent IE adhesion to hCMEC/D3

4.4. Methods

Routine HBEC and IE culture, IE staining for PfEMP1 prevalence estimation by IFA and flow cytometry. IE panning to increase binding to HBEC, recombinant protein spot binding assays and HBEC binding inhibition methods are described in chapter 2. The methods are summarised in Figure 4.1.

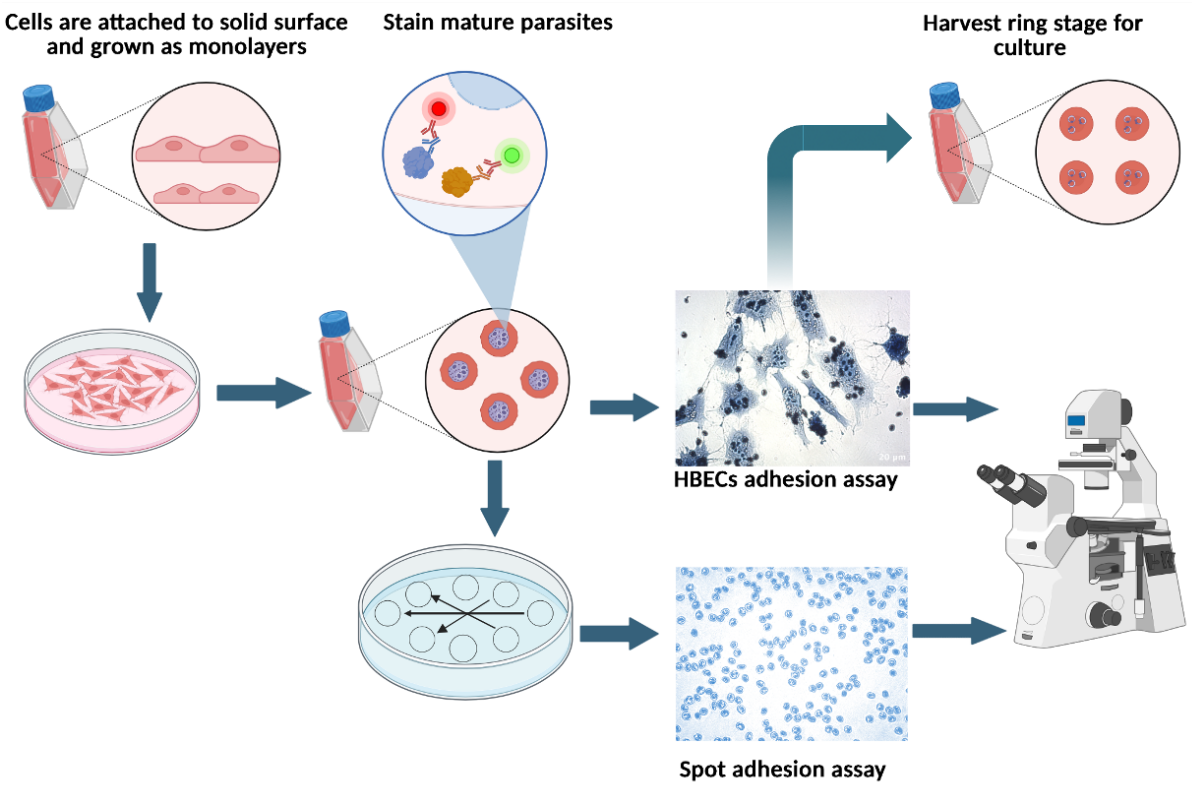


Figure 4-1. Illustration of IE panning, HBEC and spot adhesion assays. hCMEC/D3 cells are grown in a culture flask. Confluent cells are detached by trypsinisation and reseeded in a petri dish. In parallel IE are grown in a culture flask to reach the mature stage (trophozoites/schizont). In the panning assay, mature IEs are incubated

with confluent HBECs in a petri dish. After incubation and washing, adherent IEs to HBEC cells are supplied with complete parasite culture medium and maintained in culture overnight. The following day non-adherent erythrocytes including IE ring stages are collected and returned to routine parasite culture. When the parasites have grown back to high parasitaemia (>5%), the HBEC panning/selection method is repeated. Multiple rounds of panning are needed to select IEs to bind well to HBEC in vitro. A similar method of co-incubating IE with HBEC in a petri dish followed by washing to remove non-adherent cells is used in the HBEC adhesion assay to investigate the ability of antibodies and soluble proteins to inhibit binding. To quantify the number of IE adhering to 50/100 HBEC cells, adherent IEs and HBEC cells are fixed and stained with Giemsa and counted using an inverted microscope. To determine which individual receptors HBEC-selected IEs can bind, I used a spot adhesion assay. Mature IEs are incubated with recombinant C1QBP/candidate receptors spotted on a petri dish in duplicate. After incubation and washing to remove non-adherent cells, adherent IEs to recombinant proteins are fixed and stained with Giemsa and counted per field using an inverted microscope. The diagram is crafted with BioRender.com.

4.5. Results

To investigate the role of C1QBP in cytoadhesion of DC8 and non-DC8 expressing parasite lines, I first selected IEs on resting hCMEC/D3 and monitored the expression of specific PfEMP1 variants using IFA and flow cytometry. Selected IEs expressing predominantly the PfEMP1 variants of interest were used in static spot adhesion assays to determine which individual receptors the IEs can bind. To determine if C1QBP plays a role in IE cytoadhesion to HBEC, I performed a HBEC-static inhibition assay.

4.5.1. Assorted IEs selected to exhibit a particular PfEMP1 variant

To obtain a homogeneous parasite population expressing a particular PfEMP1 variant, I selected assorted IE expressing (IT4VAR19, HB3VAR03, and 9197VAR27) on hCMEC/D3. I also selected a rosetting parasite line expressing (PfKE08.g501/9605VAR2) to get a parasite population with high rosetting frequency. For experiments carried out with these parasite lines in this study, the percentage of HBEC-binding IE in cultures exhibiting the PfEMP1variants predominantly transcribed or rosetting frequency is shown below (Table 4-1).

Table 4-1: Details of parasite lines and PfEMP1 variants expressed by IE used in this study.

Genotype	**Origin**	**Predominant *var* gene transcribed (Domain cassette)**	**Percentage of IE expressing the PfEMP1 of interest/ rosetting**	**Source**
IT4	South-East Asia	*IT4var19* (DC8)	50-93	(Claessens et al., 2012)
HB3	Honduras	*HB3var03* (DC13)	70-86	(Claessens et al., 2012)
PfKE08 (aka 9605VAR2)	Kenya	*Co-expresses pfke08.g502 (DC11, rosetting variant) pfke08.g501*(DC8)	61-74	(McLean et al., 2025)
PC0053-C.G96 (aka 9197VAR27)	Kenya	*pc0053-C.g96*(DC8)	50-78	(McLean et al., 2025)

Parasite expressing IT4VAR19 was used because it is a HBEC-binding DC8-expressing line shown previously to bind to C1QBP in spot binding assays (Azasi et al., 2018; Claessens et al., 2012), and therefore acts as a positive control. Parasite expressing HB3VAR03 was used because it is a HBEC-binding DC13-expressing line, expected from previous studies to bind EPCR and ICAM1 (Avril et al., 2016; Lennartz et al., 2017) but not C1QBP (Janes et al., 2011), and therefore acts as a negative control. Two other recent culture-adapted DC8-expressing lines (PfKE08) and (9197VAR27) (McLean et al., 2025) were also used to investigate the hypothesis that C1QBP binding is a property of all IEs expressing DC8 PfEMP1 variants.

All parasite lines contain subpopulations of parasites that encode other binding variants, because transcriptional switching between *var* genes happens *in vitro*, away from the starting variant (Smith et al., 1995; Zhang et al., 2022). For all IE expressing (IT4VAR19, HB3VAR03, and 9197VAR27) selected on hCMEC/D3, I carried out repeated rounds of panning. I observe that IE binding to HBEC increases proportionally to the number of panning (Figure 4-2).

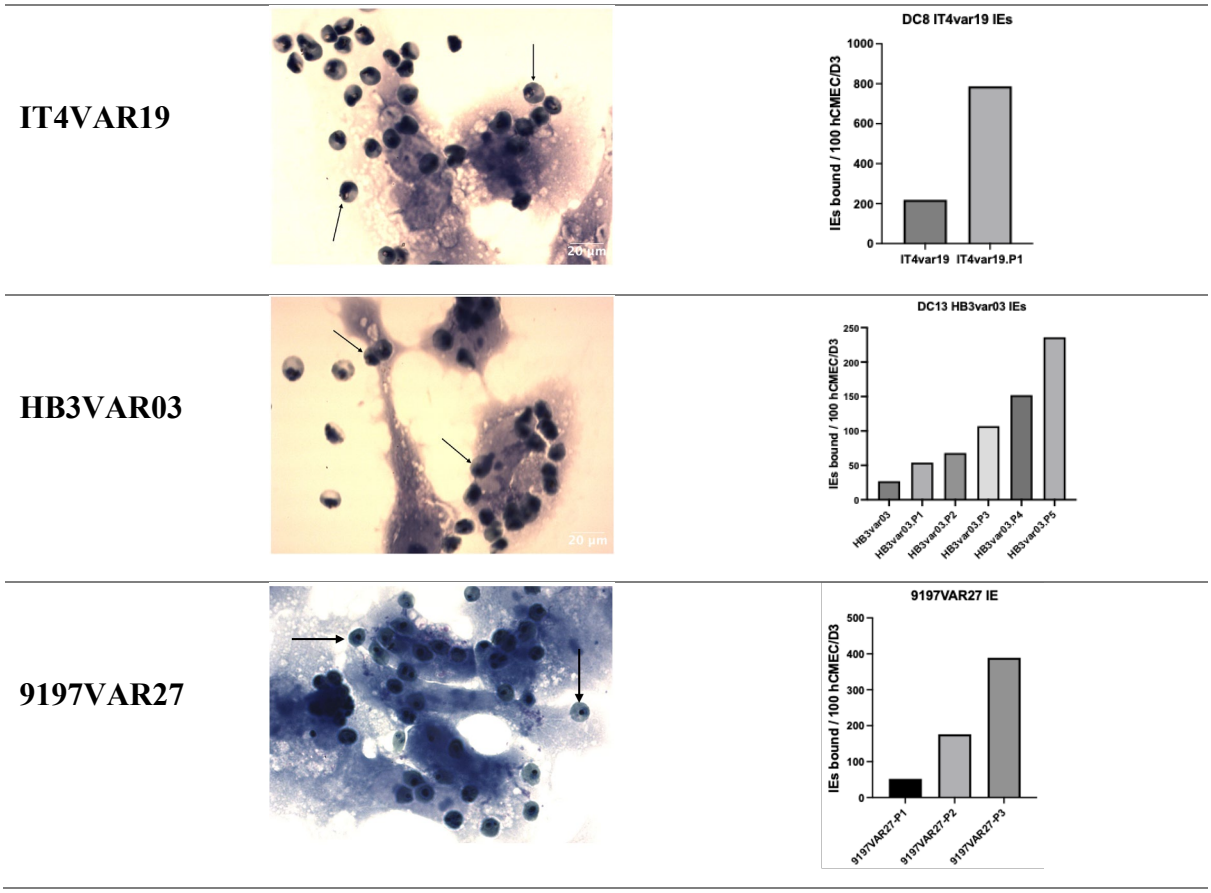
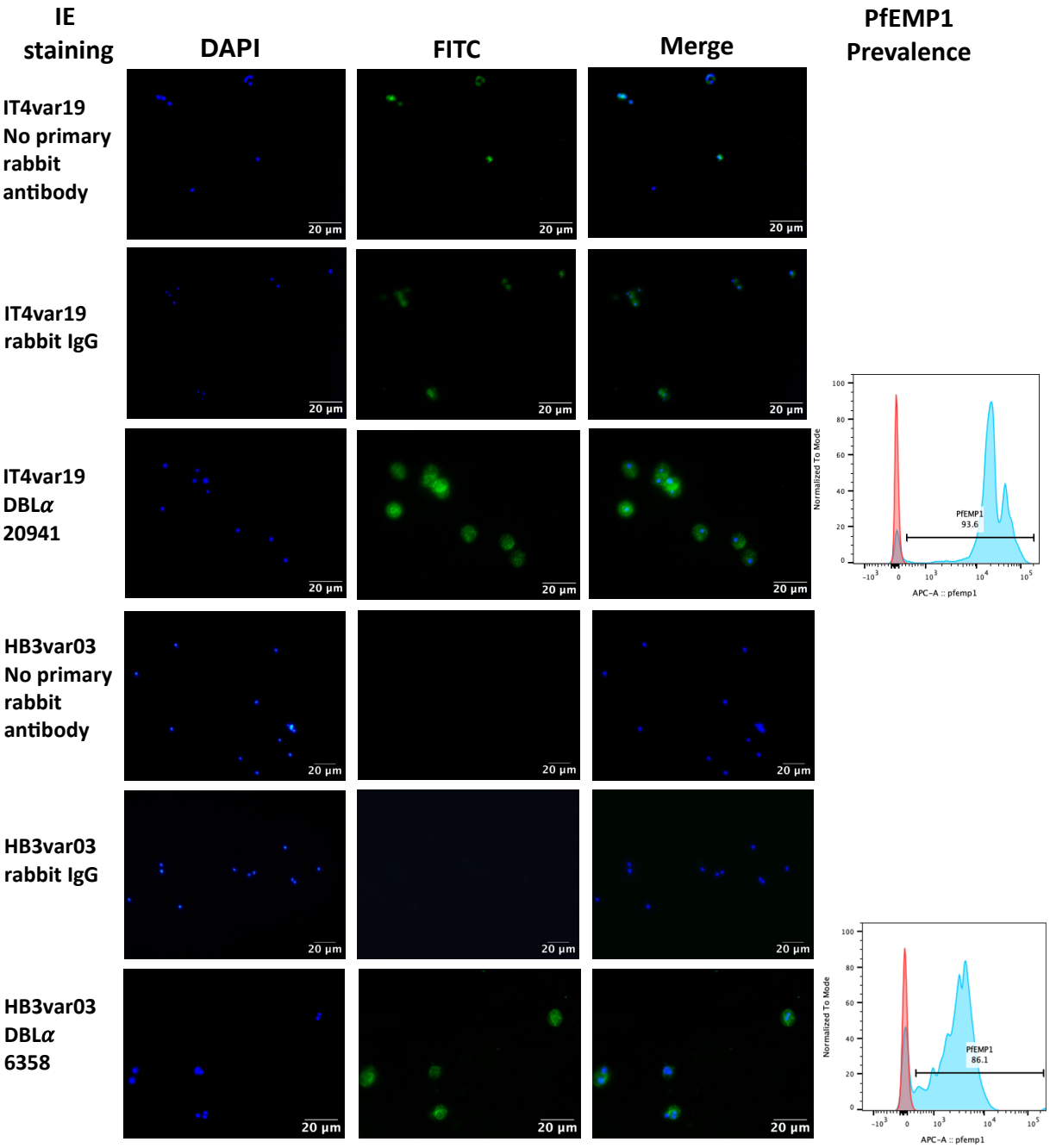


Figure 4-2: IEs panned on hCMEC/D3. IT4VAR19-IE (first row), HB3VAR03-IE (second row), and

9197VAR27-IE (third row) binding to hCMEC/D3 tested under static conditions are displayed. Each small circular cell represents mature IE incubate on large hCMEC/D3 confluent cells in petri dishes for 75 minutes at 37°C with gentle hand rocking every 30 minutes. Petri dishes were washed at least five times with incomplete DMEM-F12 Ham without sodium bicarbonate until all nonadherent cells were removed. Adherent IE cells (showed by black arrows) were fixed with glutaraldehyde 2% in PBS for at least 30 minutes and stained with 5% Giemsa for 10 minutes. Adherent IEs were counted over 100 hCMEC/D3. All pictures were taken at x1000 magnification with a scale bar of 20μm. P1 means panning round 1; P2 panning round 2 till P5 panning round 5.

The expression of the variant of interest in the panned parasite lines was monitored by IFA and flow cytometry (Figure 4-3) using variant-specific antibodies. Hence, IT4VAR19-IE show 50-93% expression of the variant of interest (Figure 4-3, third row from the top) while HB3VAR03-IE exhibit 70-86% of the specific variant (Figure 4-3 B, fourth row from the bottom). For 9197VAR27-IE, the variant of interest expression by flow cytometry is 50-78% (Figure 4-3 B, first row from the bottom).

PfKE08R+ is unusual as it expresses two PfEMP1 variants simultaneously in the same IEs (McLean et al., 2025). One is a rosette-mediating variant, and the other is a DC8 (PfKE08.g501, shown in Figure 1-5). Selection of this parasite line for rosetting selects for both the rosetting variant and the DC8, which are co-expressed. During my experiments the IE rosette frequency/percentage of DC8 positive PfEMP1 was between 61-74% (Table 4-1).



Continued to next page

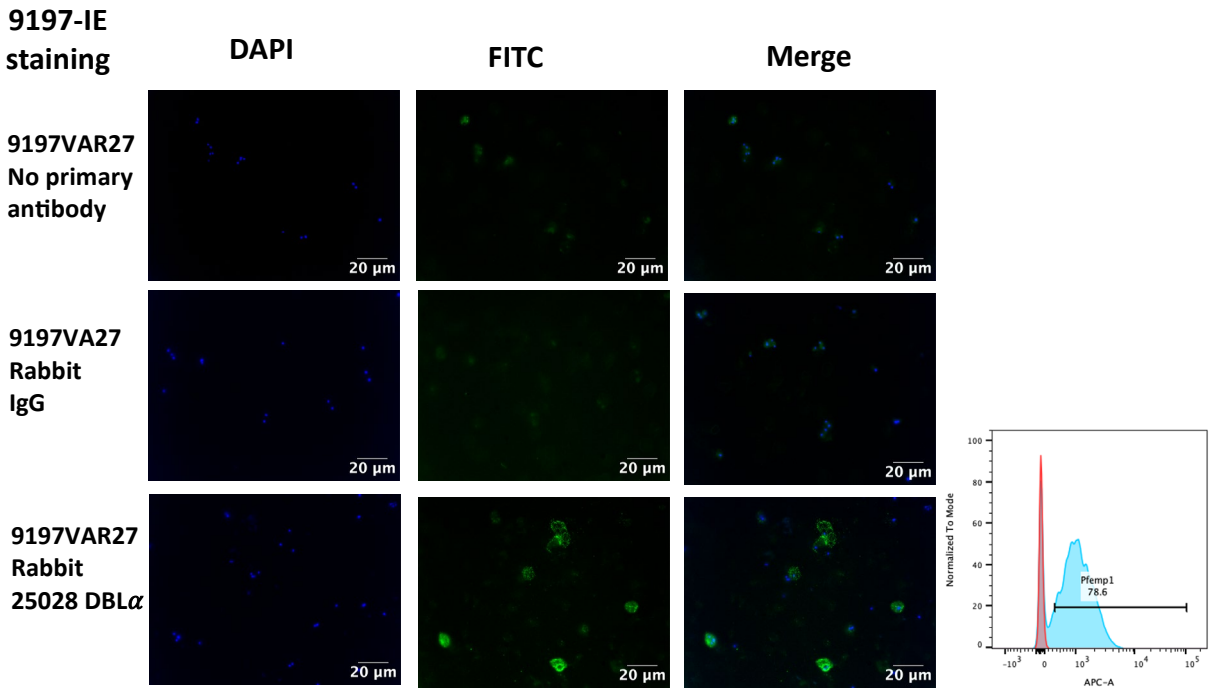


Figure 4-3. IEs stained with strain specific antibodies anti-DBLα of PfEMP1 and monitored by IFA and flow cytometer. Rabbit polyclonal antibodies to PfEMP1 recognize the surface of homologous live IEs. Rows 1 to 3 from the top show live cell stained with no primary antibody (negative control), an isotype anti-IgG (negative control), and rabbit antibody anti-20941 IT4VAR19 DBLα at 20 mg/mL tested on the homologous IT4VAR19-IE respectively. DAPI staining at 1mg/mL shows the position of IEs (blue). PfEMP1 antibody is detected by highly cross-absorbed Alexa fluor 488-conjugated anti-rabbit IgG at 1mg/mL. Specific staining of IEs is seen as punctate fluorescence over the whole of IE surface (green). The number of parasites stained with PfEMP1 antibody were counted over 100 RBCs during each panning on hCMEC/D3. Rows 4 to 7 from the bottom show live cell stained with rabbit antibody anti-6358 HB3var03 DBLα at 20 mg/mL, an isotype anti-IgG (negative control) and no primary antibody (negative control) tested on the homologous HB3VAR03-IE respectively. Rows 1 to 3 from the bottom show live cell stained with rabbit antibody anti-25028 9197VAR27 DBLα at 20 mg/mL, an isotype anti-IgG (negative control) and no primary antibody (negative control) tested on the homologous 9197VAR27-IE respectively. **Flow cytometer analysis. Third row from the top,** IT4VAR19-IE was stained with 20 mg/mL of rabbit 20941 polyclonal anti-IT4VAR19 DBLα IgG1 (blue) or an isotype rabbit IgG-negative control (Orange) followed by Alexa Fluor 647-goat anti-rabbit IgG at 1mg/mL. **Fourth row from the bottom,** HB3VAR03-IE was stained with 20 mg/mL of rabbit 6358 polyclonal anti-HB3VAR03 DBLα IgG1 (blue) or an isotype rabbit IgG-negative control (Orange) followed by Alexa Fluor 647-goat anti-rabbit IgG at 1mg/mL. **First row from the bottom,** 9197VAR27-IE was stained with 20 mg/mL of rabbit 25028 polyclonal anti-9197VA427 DBLα IgG1 (blue) or an isotype rabbit IgG-negative control (Orange) followed by Alexa Fluor 647-goat anti-rabbit IgG at 1mg/mL.

4.5.2. DC8 expressing IEs bind to recombinant C1QBP in static adhesion assay

To test whether IEs expressing (DC8) and non-DC8 PfEMP1(DC13) adhere specifically to soluble C1QBP or other recombinant proteins, I performed static binding assays. A higher frequency of bound hCMEC/D3-selected IE to soluble C1QBP or a particular recombinant protein compared to the negative control (PBS) would indicate that C1QBP may play a role in mediating IE cytoadherence and microvascular sequestration during CM.

DC8 expressing IT4VAR19-IEs derived from one round of panning on hCMEC/D3 significantly bind to host C1QBP, CD36, CD31, EPCR, TSP, and VCAM1 (Figure 4-4 A). However, DC13 expressing IEs derived from five rounds of panning on hCMEC/D3 do not show any binding to C1QBP or any known candidate receptors for human endothelial cells apart the positive control CD36 (Figure 4-4 B). The binding of both these lines to CD36 is likely to be due to subpopulations of parasites in these cultures exhibiting different PfEMP1 variants of group B and C type, identified to interact with CD36 through their CIDRα2-6 domain (Hsieh et al., 2016) (Figure 4-4 A and B). The lack of HB3VAR03-IE binding to EPCR and ICAM1 does not agree with some previously published data (Lennartz et al., 2017), but is consistent with previous work from the Rowe lab (Azasi et al., 2018; Claessens et al., 2012).

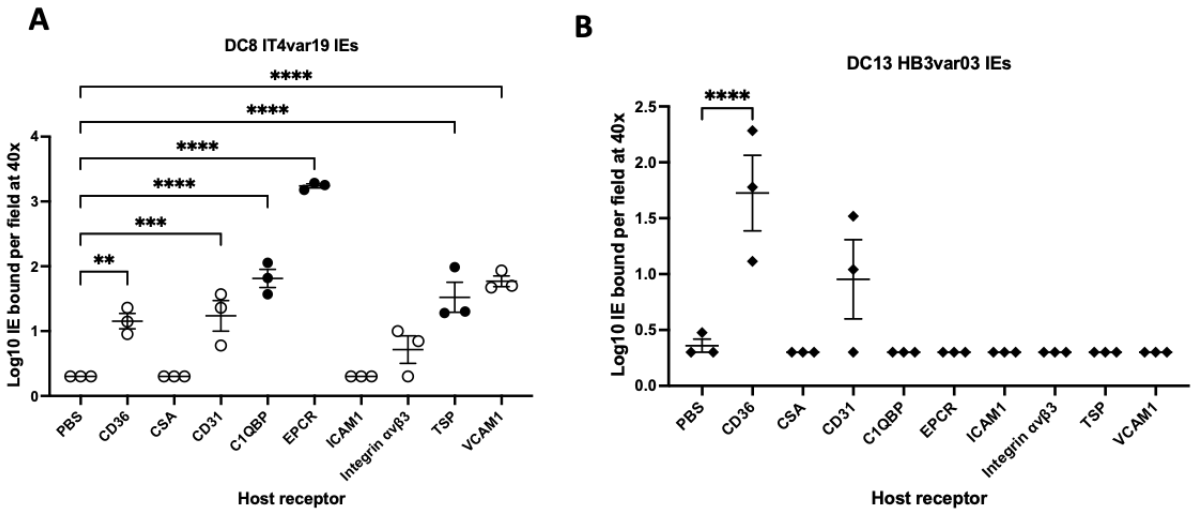


Figure 4-4. DC8 IT4var19 and DC13 HB3var03 IEs bind to C1QBP. IT4var19 IEs (A) and HB3var03 IEs (B) bind recombinant proteins adsorbed onto plastic petri dishes tested under static conditions is displayed. Mature IEs incubate on soluble proteins at 50 μg/mL in petri dishes for 1 hour at 37°C with gentle hand rocking every 12 minutes. Petri dishes were washed at least five time with RPMI 1640 without sodium bicarbonate until all nonadherent cells were removed. Adherent IE cells were fixed with glutaraldehyde 2% in PBS for at least 30 minutes and stained with 5% Giemsa for 10 minutes. **A-)** The black circles are IEs that stained positive with DC8 PfEMP1 homologous antibodies, and the white circles did not stain positive with the DC8 PfEMP1 antibodies. **A and B)** Data for each spot binding represents an independent experiment and it is also the average of at least six independent fields reading (each dot). The number of IE bound per field was determined by an inverted microscope. PBS was used as a negative control and CD36 as a positive control. Data were Log (10) transformed before to apply statistics. The difference in mean binding values compared with the PBS negative control from n ≥ 3 independent experiments was analyzed by one-way ANOVA with Dunnett's multiple comparison test. *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001. Graph A and B have different y axis scale.

To determine whether IEs that bound to recombinant C1QBP and other potential receptors expressed the DC8 PfEMP1 or are due to subpopulations that have switched ot other variants, I carried out immunofluorescence staining on the bound cells using variant-specific DBLα antibodies. IT4VAR19-IEs binding to recombinant C1QBP, EPCR and TSP were confirmed to exhibit the PfEMP1 variant DC8 (Figure 4-4, rows 1, 2, and 3 from the top), whereas CD36, CD31 and VCAM1 bound IEs did not express the PfEMP1 variant DC8 (Figure 4-4, rows 1, 2, and 3 from the bottom). Taken together these results show that IT4VAR19 DC8-expressing IEs adhered to recombinant C1QBP while HB3VAR03 DC13-expressing IEs did not. The IT4VAR19 DC8- expressing IEs also bound to TSP and EPCR.

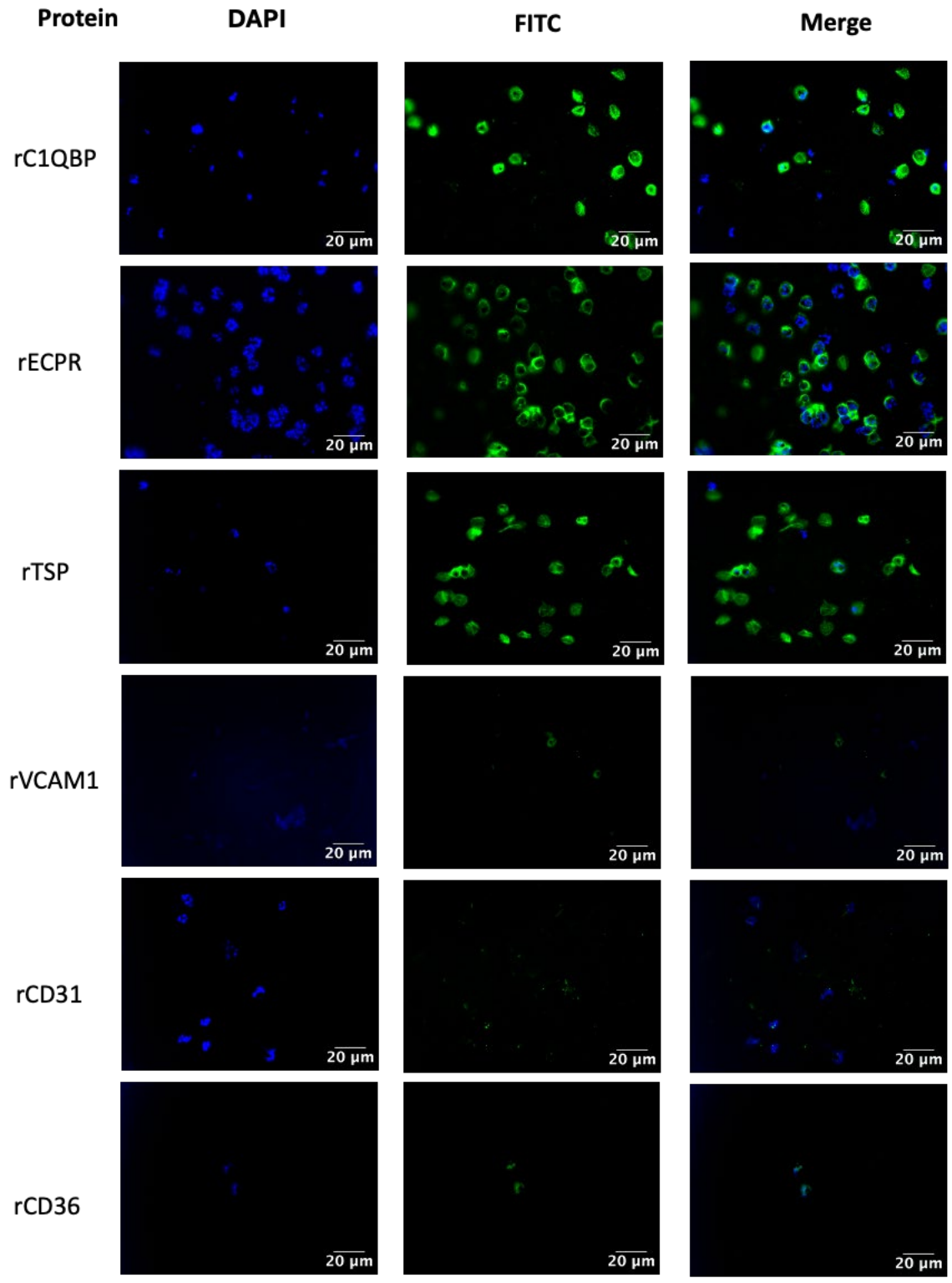


Figure 4-5. Staining of bound IEs from static receptor-binding assay with rabbit polyclonal IgG to IT4var19 DBLα. Bound IEs from the IT4var19 culture were identified by staining parasite nuclei with 1 mg/mL DAPI (blue). IEs bound to C1QBP, EPCR and TSP shown green punctate surface staining with 20 mg/mL rabbit polyclonal IgG to IT4var19 DBLa, followed by a 1:500 dilution of Alexa fluor 488 goat anti-rabbit IgG, displaying that the bound cells expressed this specific variant (green). IEs bound to VCAM-1, PECAM-1 and CD36 did not stain with the IT4var19 variant-specific antibodies, suggesting that other variant types in culture mediate VCAM-

1, PECAM-1 and CD36 binding. All pictures were taken at 100X with a scale bar of 20µm. Results showed here are representative of at least two independent experiences. r means recombinant.

4.5.3. C1QBP binding is not a property of all IEs expressing DC8 PfEMP1 variants

To determine whether C1QBP binding is a common feature of all IEs expressing DC8 PfEMP1, I investigated the ability of 9197VAR27 and PfKE08/9605R+ to interact with soluble C1QBP. In a recent study I clearly demonstrated the ability of 9605R+ IEs expressing PfEMP1 DC8-like to interact with EPCR and ICAM1 (McLean et al., 2025). However, the possibility of 9197VAR27 and 9605R+ IEs expressing PfEMP1 DC8-like variant to interact with C1QBP was not examined in that initial study. I therefore investigated the ability of 9197VAR27 and 9605R+ IEs to interact with C1QBP in the absence and presence of rosette disruption using static adhesion assays. The presence of rosettes can interfere with binding to receptors on plastic dishes; therefore the inclusion of rosette disruption conditions was to ensure that meaningful binding interactions were not missed for this reason. I found that 9197VAR27 and 9605R+IEs did not bind to C1QBP (Figure 4-6 A and B). As see previously (McLean et al., 2025), 9605R+ IEs did significantly adhere to EPCR and ICAM1, with higher binding after rosette disruption (Figure 4-6 B). These results show that 9197VAR27 and 9605R+ IEs do not interact with C1QBP despite the presence of the DBLβ12 DC8 domain in their PfEMP1 architecture.

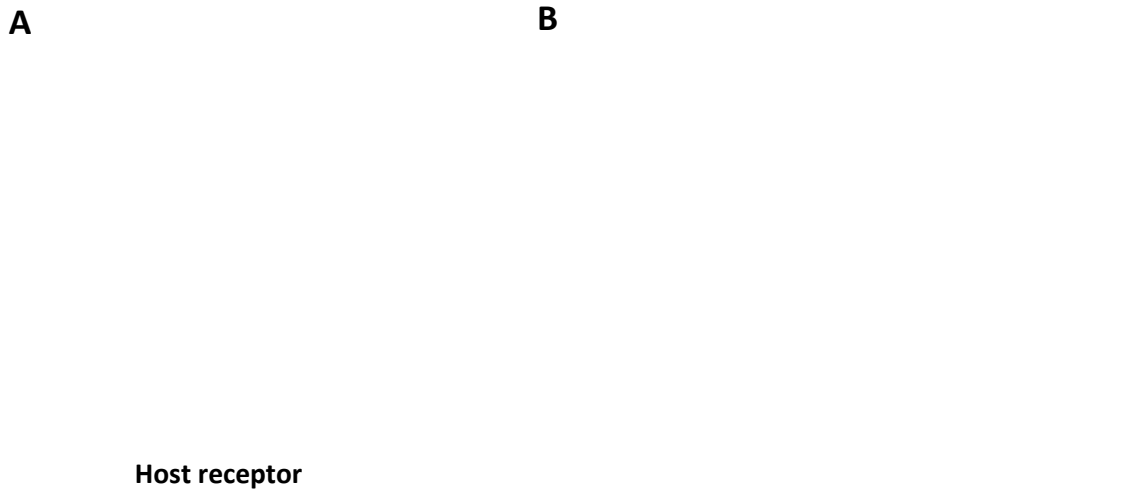


Figure 4-6. Spot binding assay of IEs to recombinant C1QBP and other receptors. A-) 9197VAR27-IE binding (white square) to recombinant proteins. **B-)** Adhesion of 9605-IE was determined with (white circles) and without (black circles) prior rosette disruption with antibodies to g502 NTS DBLα. Each square/dot is the average

of six randomly chosen field per dish and assay. The number of IE bound per field was determined using an inverted microscope with a 40X objective and six fields were counted per dish. Data were Log (10) transformed before to apply statistic. The difference in mean binding values compared with the PBS control from n≥ 3 independent experiments was analyzed by one-way ANOVA with Dunnett's multiple comparison test. The same receptor in presence and absence of rosette from n ≥ 3 independent experiments was analyzed by two-tailed paired t tests corrected with Holm-Sidak multiple comparisons test. *P < 0.05 indicates statistical significance.

4.5.4. C1QBP is not a major receptor that mediates IT4VAR19 IEs adhesion to HBEC

To determine if C1QBP plays a role in promoting IT4VAR19 IE adhesion to hCMEC/D3, I conducted HBEC binding-inhibition assays. hCMEC/D3 cells grown with 10% pooled normal human plasma to allow C1QBP to associate with the membrane were preincubated for 1 hour with specific antibodies to block cytoadhesion receptors (e.g. monoclonal/polyclonal antibody to human C1QBP) or 1%BSA (control). Similarly, IT4VAR19 IEs were pretreated with recombinant proteins such as C1QBP, EPCR and ICAM1. I found that preincubation of hCMEC/D3 with antibodies to human C1QBP and ICAM1 had no effect on the interaction between IEs and hCMEC/D3, whereas a monoclonal antibody to human EPCR did significantly reduced cytoadhesion (Figure 4-7 A). Similarly, preincubation of IT4VAR19 IEs with recombinant C1QBP and ICAM1 had no effect on binding, whereas recombinant EPCR significantly reduced cytoadhesion (Figure 4-7 B), as shown previously (Azasi et al., 2018). These observations do not support the hypothesis that C1QBP is a major receptor for IT4VAR19 IEs adhesion to hCMEC/D3.

A **B**

Figure 4-7. DC8 IT4var19-IE binding to hCMEC/D3 is not mediated by host C1QBP. (A) hCMEC/D3 were preincubated with antibodies to C1QBP, EPCR or ICAM1 or (B) DC8 IT4var19-IE were preincubated with recombinant C1QBP, EPCR or ICAM1 before static adhesion assay as described in the methods. Each data point is from an independent experiment, and the mean and SEM are shown. Data were Log (10) transformed before statistical analysis. The difference in mean values compared with control (no preincubation with antibodies or recombinant protein) was analysed by one-way ANOVA with Dunnett's multiple comparison test. *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001.

4.6. Discussion

4.6.1. Summary of main findings

The main objective of this chapter was to determine whether C1QBP promotes IE expressing PfEMP1 DC8 binding to hCMEC/D3. IEs (IT4VAR19, 9197VAR27, and 9605R+/PfKE08) were selected for HBEC-binding or rosetting phenotype to get a homogeneous parasite population expressing PfEMP1 DC8. All IE expressing PfEMP1 DC8 interact with EPCR but not C1QBP. Only IT4VAR19-IE significantly bind to C1QBP, however monoclonal and polyclonal antibodies anti-human C1QBP do not prevent IT4VAR19-IE cytoadhesion to hCMEC/D3. Overall, C1QBP is not a major endothelial cell receptor for IE adhesion to HBEC.

4.6.2. HBEC selection of IE expressing DC8 and non-DC8 PfEMP1 variants

Parasite lines derived by panning on resting hCMEC/D3 or selected for rosetting that express DC8 PfEMP1 (IT4VAR19, 9197VAR27, and PfKE08) and non-DC8 PfEMP1 (HB3VAR03) were used to investigate IE binding to C1QBP. They were chosen for some because the PfEMP1 DBLβ12 domain of 3D7_PDF0020c IE was identified as the main ligand that mediate IE adhesion to host C1QBP (Magallon-Tejada et al., 2016). The parasite expresses PfEMP1 variant that has been previously associated with CM and other forms of SM (Avril et al., 2012; Claessens et al., 2012; Lavstsen et al., 2012).

The panning predominantly increases the expression of a particular PfEMP1 variant on the surface of enriched IEs and their adhesion frequency to HBECs (Figure 4-2 A and B). Before HBEC selection IEs (IT4VAR19, HB3VAR03 and 9197VAR27) showed lower PfEMP1 prevalence and binding frequency to hCMEC/D3. After selection, IEs exhibited a higher PfEMP1 prevalence and binding frequency to hCMEC/D3.These observations are consistent with previous studies (Avril et al., 2012; Azasi et al., 2018; Claessens et al., 2012; Claessens & Rowe, 2012).

This panning method also has been used to select IEs on diverse endothelial cells (primary and immortalised HBEC) and all studies have led to the dominant expression of parasite ligands DC8 and DC13 PfEMP1, associated with SM (Avril et al., 2012; Claessens et al., 2012). Selected IEs on HBEC can also help to identify major receptors involved in cytoadhesion (Claessens & Rowe, 2012).

4.6.3. IEs expressing PfEMP1 DC8 bind to C1QBP

In spot binding assays, HBEC selected IE predominantly expressing DC8 PfEMP1 bind to C1QBP, CD36, CD31, EPCR, TSP, and VCAM1 (Figure 4-4 A). However, only IEs binding to C1QBP, EPCR and TSP are stained positive to antibody anti-IT4VAR19-DBLα in IE bound cells IFA (Figure 4-5). IE binding to C1QBP was also described in previous studies (Azasi et al., 2018; Claessens et al., 2012) but the binding was not significant because precedent works did not Log transform their data. By Log transforming my IE binding data before to use the one-way ANOVA, I found that IT4VAR19-IE significantly bind to C1QBP. Log transforming data is important because it can make the distribution more normal and validates the use of a statistic parametric test.

DC13-expressing IE did not bind soluble C1QBP and other brain endothelial candidate receptors but significantly bound to recombinant CD36 (Figure 4-4 B). This result agrees with prior works (Azasi et al., 2018; Claessens et al., 2012). Major study has used recombinant protein to show that IE expressing DC13 play a crucial role in the adhesion of IE to HBEC (Turner et al., 2013). But subsequent studies have found that IE expressing native PfEMP1 DC13 did not interact with candidate receptors associated with SM (Azasi et al., 2018). Therefore, there is a need to search for additional receptor involved in IE sequestration.

DC8-expressing IEs also significantly adhere to C36, CD31, and VCAM1 but they did not stain positive for antibody anti-IT4VAR19 DBLα. This adhesion of DC8 and DC13-expressing IEs to recombinant CD36 can be explained by the presence of multiple variants in parasite isolates with different binding phenotypes because of PfEMP1 antigenic variation (Smith et al., 1995; Zhang et al., 2022). These findings are in line with previous results (Avril et al., 2013; Azasi et al., 2018; Claessens et al., 2012).

Prior studies also have associated the cytoadhesion phenotype of IE-C1QBP to clinical severe malaria presentations like SMn and multiple seizures (Mayor et al., 2011) and found that IE (3D7_PDF0020c) expressing a DC8-like PfEMP1 used DBLβ12 to interact with C1QBP (Magallon-Tejada et al., 2016). Therefore, it is important to investigate whether all parasite expressing DC8 PfEMP1 bound to C1QBP.

4.6.4. Other IEs expressing DC8 PfEMP1 did not interact with C1QBP

Unexpectedly well characterised IEs (9197VAR27 and PfKE08/9605R+) which contain DBLβ12 in their PfEMP1 architecture did not interact with C1QBP. Our static adhesion study suggests that 9197VAR27-IE and 9605R+ IE bound to EPCR but not C1QBP despite having a DBLβ12 in their PfEMP1 molecular structure. However, the study that identify IE DBLβ12 as the binding partner for C1QBP performed an ELISA-based binding assays (Magallon-Tejada et al., 2016). Further study carried out computational simulation to characterise the interaction between 3D7_PDF0020c DBLβ12 and C1QBP (Bakri et al., 2021). They identified 21 amino acid residues in DBLβ12 that interact with C1QBP and argued that the binding site of ICAM1 and C1QBP are in different DBL domains. IEs exhibiting DBLβ1, DBLβ3 and DBLβ5 from PfEMP1 group A, B, and C are recognised to interact with ICAM1 but not DBLβ12 (Janes et al., 2011; Lennartz et al., 2017; Lennartz et al., 2019).

However, 9605R+ IE displayed a dual binding phenotype, EPCR and ICAM1 (Figure 4-6 B) despite the presence of DBLβ12 in their PfEMP1 architecture. This result agrees with recent adhesion binding assays (McLean et al., 2025) but challenged previous expectations (Janes et al., 2011; Lennartz et al., 2017; Lennartz et al., 2019). A plausible explanation for this discrepancy maybe the fact that 9605R+ IE belongs to group B/A and not group A, B, and C PfEMP1 based on its DBLα molecular structure, and PfEMP1 transcription direction (Rask et al., 2010). Therefore, the molecular mechanism used by 9605R+ IE DBLβ12 to interact with ICAM1 remains to be identified.

4.6.5. C1QBP did not mediate IT4VAR19 IE adhesion to hCMEC/D3

To examine that host C1QBP is an important receptor for DC8 expressing IE adhesion to hCMEC/D3, HBEC-inhibition assay was conducted. hCMEC/D3 cells were grown in 10% pooled normal human plasma to provide the possibility of soluble C1QBP to stick on the surface membrane of hCMEC/D3. hCMEC/D3 cells were preincubated with monoclonal/polyclonal antibodies anti-human C1QBP to block C1QBP proteins expressed on the surface of hCMEC/D3. While IT4VAR19 IEs were preincubated with recombinant C1QBP to block IE ligand DBLβ12 recognised to mediate C1QBP binding.

Monoclonal/polyclonal antibodies anti-human C1QBP and recombinant C1QBP did not prevent the interaction between DC8 expressing IE and hCMEC/D3 (Figure 4-7 A and B

respectively). These results are not in line with the findings of Biswas et al, who argued that mouse monoclonal antibody anti-C1QBP and recombinant C1QBP reduced in dose dependent fashion the adhesion of IE to human endothelial cells (Biswas et al., 2007). The difference in the frequency of binding inhibition between the study of Biswas et al, and the present work could be attributed to the following factors: the laboratory adapted parasite strain used in previous study was 3D7_PDF0020c rather than IT4VAR19 IE in the present study. Biswas et al, panned 3D7_PDF0020c and field isolate parasites with recombinant C1QBP in static binding assays instead of HBEC-selected IT4VAR19 IE in this study. The recombinant C1QBP spot binding selection conducted by Biswas et al, may induce a preferential high affinity interaction between 3D7_PDF0020c IE and host C1QBP. Both studies used human brain endothelial cells in inhibition assays but from different sources, HBMEC for Biswas et al, and hCMEC/D3 in the present study. Biswas et al, produced in house the recombinant protein and the mouse antibody anti-human C1QBP, whereas I have used commercial recombinant protein and antibodies anti-human C1QBP from Sino Biologicals. The affinity of different recombinant proteins and antibodies in preventing IE cytoadhesion may differ.

However, the monoclonal antibody anti-human EPCR and recombinant EPCR strongly inhibit the interaction between DC8-expressing IE and hCMEC/D3 (Figure 4-7 A and B respectively). This result agrees with previous studies (Azasi et al., 2018; Turner et al., 2013). I also observed that the combination of monoclonal antibody anti-human EPCR and anti-human ICAM1 significantly reduce the cytoadhesion of DC8-expressing IE, but the frequency of binding inhibition was inferior to the monoclonal antibody anti-human EPCR alone (Figure 4-7 A), suggesting the absence of synergistic action between EPCR and ICAM1 in mediating DC8-expressing IE binding to hCMEC/D3 in this study.

4.7. Conclusion

The data in this chapter indicate that not all DC8-expressing IEs bind to C1QBP. IT4VAR19-IEs do bind to C1QBP in spot binding assays but antibody to human C1QBP and recombinant C1QBP do not block the cytoadhesion of IT4VAR19-IEs to hCMEC/D3. Hence C1QBP is not the major endothelial cell for the parasite lines studied here.

5. Chapter V: Discussion

5.1. Introduction

Previous studies suggest C1QBP as a potential receptor for IE sequestration during CM (Biswas et al., 2007). This cytoadhesion phenotype has been associated with other forms of SM but not CM (Mayor et al., 2011). HBEC-selected IE adhesion to C1QBP is missing in the literature. Therefore, the main aim of this thesis was to elucidate the role of C1QBP in mediating HBEC-selected IE adhesion to hCMEC/D3. To address this, hCMEC/D3 culture was used as a model to examine the cytoadherence of IEs associated with severe and CM. Firstly, hCMEC/D3 was authenticated as an endothelial cell origin and used to determine the cellular localisation of C1QBP and other candidate receptors by IFA and fluorescence microscopy. Resting and TNF-α activated hCMEC/D3 did not show surface expression of C1QBP (Figure 3-3). However, preincubated hCMEC/D3 with recombinant C1QBP and normal human plasma (containing soluble C1QBP) enhanced the surface detection of C1QBP (Figure 3-6 and 3-7). The same result was obtained for another immortalised human brain endothelial cells (HBEC-5i) (Figure 3-11 and 3-12). In contrast, primary human endothelial cells (HBMEC, HPMEC, and HDMEC) constitutively exhibited C1QBP (Figure 3-14). These results indicate that immortalised endothelial cells may not fully express the same receptor as primary endothelial cells.

Secondly, four laboratory adapted parasite lines (IT4VAR19, HB3VAR03, 9197VAR27 and PfKE08) were successfully selected for cytoadhesion to hCMEC/D3 and rosetting respectively. This selection led to a significant increase of IT4VAR19, HB3VAR03, 9197VAR27 IE PfEMP1 variant expression and their binding frequency to hCMEC/D3 (Figure 4-2) as well as the rosetting frequency of PfKE08. The possibility of C1QBP to mediate IE sequestration in human cerebral vascular beds was investigated by using a combination of static adhesion assays on recombinant proteins as well as binding inhibition assays on cultured hCMEC/D3 with recombinant C1QBP and antibodies to human C1QBP. IT4VAR19 IE showed significant binding to C1QBP, TSP, and EPCR (Figure 4-3). However, antibodies to human C1QBP did not prevent the interaction between IEs and hCMEC/D3 (Figure 4-6). These findings showed that IE binding to hCMEC/D3 is not C1QBP dependent.

The implications of these findings are discussed with relevant knowledge in CM research.

5.2. Is hCMEC/D3 a suitable cell line for *P. falciparum* cytoadhesion research?

hCMEC/D3 cell line has been characterised by Weksler et al (Weksler et al., 2005), as a reliable model for studies of BBB function, responses of brain endothelium to inflammatory and infection stimuli, and the interaction of brain endothelium with other cells (Weksler et al., 2005). hCMEC/D3 was tested positive for expression of endothelial markers such as PECAM1/CD31 and vWF, suggesting its endothelial nature. hCMEC/D3 cell line also exhibited adhesion molecule associated with IE sequestration, including ICAM1 and VCAM1 only when cells were activated by TNF-α (Weksler et al., 2005). However, very few studies have used hCMEC/D3 to investigate the molecular mechanism of IE cytoadherence (Jambou et al., 2010; Zougbede et al., 2011).

5.2.1. hCMEC/D3 authenticates as a human endothelial cell nature

To validate the endothelial nature of hCMEC/D3, I labelled hCMEC/D3 for the expression of specific markers of human endotheial cells. As expected hCMEC/D3 showed positive surface expression for PECAM1/CD31, intracytoplasmic staining for vWF and DIL-Ac-LDL, and a negative staining for SMA (Figure 3-2). SMA is a negative control for endothelial cell but provides positive staining for fibroblasts. These results indicate that hCMEC/D3 is from human endothelial origin, and results are supported by prior researches. These reseraches also argued that hCMEC/D3 form a confluent monolayer with tight intercellular junctions and resembles the in vivo BBB (Weksler et al., 2013; Weksler et al., 2005).

5.2.2. C1QBP expression by human endothelial cells

I further assessed the cellular localisation of C1QBP on hCMEC/D3 with IFA and fluorescence microscopy. I found that C1QBP is not constitutively express on hCMEC/D3 (Figure 3-3). In contrast to previous study that used flow cytometry and MFIs to describe C1QBP as a HBEC surface membrane receptor (primary and immortalised HBEC) (Biswas et al., 2007). This suggests that flow cytometry alone is not sufficient to characterise the cellular localisation of C1QBP on hCMEC/D3. Hence the combination of flow cytometry and IFA may contribute to better characterise HBEC cell surface receptor. The disparity in results is described in section 3.6.3. My finding raises a concern about using hCMEC/D3 to study the role of C1QBP in IE

cytoadhesion. However, another study claimed the possibility of soluble C1QBP to bind to HBEC and provides additional surface receptors (Ghebrehiwet et al., 2014).

To test the ability of soluble C1QBP to attach on hCMEC/D3, I preincubated soluble C1QBP with a confluent monolayer of hCMEC/D3 before IFA. I found that some hCMEC/D3 cells showed surface expression of C1QBP (Figure 3-6). This result highlights the ability of soluble C1QBP to compensate the deficit of hCMEC/D3 for C1QBP expression.

To confirm whether this applies to other immortalised HBEC, I stained HBEC-5i (Dorovini-Zis et al., 2011) in both resting and activated conditions for C1QBP expression. I found that C1QBP is not natively express by HBEC-5i either in resting or activated conditions (Figure 3-11). Therefore, I preincubated confluent monolayer of HBEC-5i with soluble C1QBP before IFA. I observe surface membrane expression of C1QBP in resting and activated HBEC-5i. This result attests that both immortalised HBEC lines do not constitutively express C1QBP but can show C1QBP surface expression when they are preincubated with soluble C1QBP.

To test if the same happens for primary HBECs, I have tested the capacity of primary HBMEC to constitutively express C1QBP. I found that resting and TNF-α activated HBMEC natively exhibit a homogeneous surface expression of C1QBP (Figure 3-15). The same results occur with other primary endothelial cells, HPMEC, and HDMEC (Appendix, Figure 6-3 and 6-6). Therefore, All the primary endothelial cells tested here constitutively exhibit surface expression of C1QBP in resting and activated conditions.

The cellular localisation of C1QBP remains a matter of dispute in the existing literature. The discrepancies between research groups is described in section 3.6.3. Some have desmontrated surface membrane expression of C1QBP (Andrews et al., 1981; Daha et al., 1988; Ghebrehiwet et al., 1994) and others claimed intracellular localisation of C1QBP (Dedio et al., 1998; Dedio & Muller-Esterl, 1996; van den Berg et al., 1997). My study has demonstrated stringent difference between surface expression of C1QBP between immortalised and primary endothelial cells. However, unlike the classical cell surface receptor, C1QBP is missing a consensus motif for transmembrane domain and a GPI (glycosylphosphatidylinositol) anchor (Ghebrehiwet et al., 1994). Hence, C1QBP may attach to cell surface membrane from soluble C1QBP (Ghebrehiwet et al., 2014) and likely by adhesion with transmembrane protein such as

the interaction between fibrinogen and ICAM1(Cheung et al., 1991), HK and vitronectin to C1QBP (Phan et al., 2022) to serve as a receptor.

5.2.3. Endothelial cell receptors expressed by hCMEC/D3

Endothelial cells surface receptors play a crucial role in the adhesion and sequestration of IE during CM. The use of *in vitro* model like hCMEC/D3 can contribute to better decipher receptors involved in CM. To date thirteen host receptor candidates have been identified to mediate IE cytoadhesion (Table 5-1).

Table 5-1: First description of receptor involved in sequestration of IE (adapted from(Rogerson & Milner Jr, 2009)).

Receptor	Reference
TSP	(Roberts et al., 1985)
ICAM1	(Berendt et al., 1989)
E-selectin	(Ockenhouse, Tegoshi, et al., 1992)
VCAM1	(Ockenhouse, Tegoshi, et al., 1992)
CD36	(Barnwell et al., 1985)
CSA	(Rogerson et al., 1995)
P-selectin	(Udomsangpetch et al., 1997)
PECAM1/CD31	(Treutiger et al., 1997)
αvβ3 integrin	(Siano et al., 1998)
Fractalkine	(Hatabu et al., 2003)
NCAM	(Pouvelle et al., 2007)
C1QBP	(Biswas et al., 2007)
EPCR	(Turner et al., 2013)

Among these above-mentioned receptors, I have found that hCMEC/D3 constitutively express ICAM1, PECAM1/CD31, VCAM1, and EPCR (Figure 3-3, 3-4, and 3-5). These receptors have been previously associated with IE cytoadhesion. However, the other nine receptors associated IE cytoadhesion were not natively express by hCMEC/D3 (Figure 3-3, 3-4, and 3-5). The difference in receptor expression with other studies has been discussed in section 3.6.7 and 3.6.8. However, I have demonstrated that C1QBP can become membrane associated to

hCMEC/D3 when added exogenously or from normal human plasma (that contains C1QBP) (Figure 3-6 and 3-7). This finding was also observed in previous study (Ghebrehiwet et al., 2014). This result provides an alternative for future studies that aim to investigate the role of C1QBP in cytoadhesion with immortalised endothelial cells.

5.2.4. hCMEC/D3-selected IE for cytoadhesion

Previous studies have used immortalised HBEC-5i and primary endothelial cells (HBMEC. HPMEC, and HDMEC) to identify the parasite ligands involved in IE sequestration and severe disease (Avril et al., 2012; Claessens et al., 2012). However, hCMEC/D3-selected IE role in cytoadhesion is rare in the literature. I selected on hCMEC/D3 well characterised parasite lines associated with different phenotype of cytoadhesion. As expexted, I found that IE expressed dominant PfEMP1 DC8 (IT4VAR19, 9197VAR27) and DC13 (HB3VAR03). These results are in line with previous studies (Avril et al., 2013; Avril et al., 2012; Azasi et al., 2018; Claessens et al., 2012). Therefore, the selection of IE on endothelial cells increases the binding between IE and HBECs. (Claessens & Rowe, 2012).

5.2.5. Role of C1QBP in IE cytoadhesion

Most available data on IE cytoadhesion are focused on laboratory adapted parasite lines (Avril et al., 2013; Avril et al., 2012; Claessens et al., 2012). Here, I investigated the role of C1QBP in IE adhesion using four laboratory adapted parasite lines (IT4VAR19, HB3VAR03, 9197VAR27 and PfKE08) selected for hCMEC/D3 binding and rosetting respectively. These parasite lines expressed either DC8 or DC13 in their PfEMP1 molecular architecture. Only IT4VAR19-IE (DC8-expressing IE) showed significant adhesion to C1QBP (Figure 4-3). The interaction between IT4VAR19-IE and C1QBP might be mediated by the DC8 DBLβ12 domain as previously suggested (Magallon-Tejada et al., 2016). Magallon et al used a parasite line named 3D7_PDF0020c to show that the DC8 DBLβ12 domain is the IE ligand that interacts with C1QBP. I tried to work with this parasite line but could not select it for adhesion to HBEC. I also tested other DC8-expressing parasite lines in the expectation that they would bind C1QBP, but only one of them (IT4VAR19-IE) did so. In static adhesion inhibition assays antibodies to human C1QBP did not significantly reduce the cytoadhesion between IT4VAR19-IEs and hCMEC/D3 (Figure 4-6). Therefore, these results do not support the hypothesis that the DBLβ12 domain of DC8 PfEMP1s always bind to C1QBP and contribute to sequestration,

and highlight the risk of generalising results from a single parasite line. This result is in line with previous work (Avril et al, 2013) showing that recombinant DBLβ12 domains are less effective than other DC8 PfEMP1 domain types at inhibiting the interaction between IE and HBEC. Therefore, C1QBP is not the major endothelial cell receptor for IE sequestration for the parasite lines used here, but the possibility remains that it might act in combination with other receptors to facilitate IE accumulation in HBECs.

5.3. Other possible roles of C1QBP in malaria host-parasite interaction

It remains possible that IE binding to C1QBP could play a role in host-parasite interaction in malaria beyond cytoadhesion, such as in complement inactivaction. For example, I proposed that the globular head of C1q (gC1q), the physiologic ligand of C1QBP is available in the plasma/serum as circulating C1 complex (gC1q, C1r, and C1s). Engagement of the gC1q with endothelial surface C1QBP (Biswas et al., 2007; Ghebrehiwet et al., 1994) may induce the activation of the C1 complex and released of gC1q, C1r, and C1s (Figure 5-1).

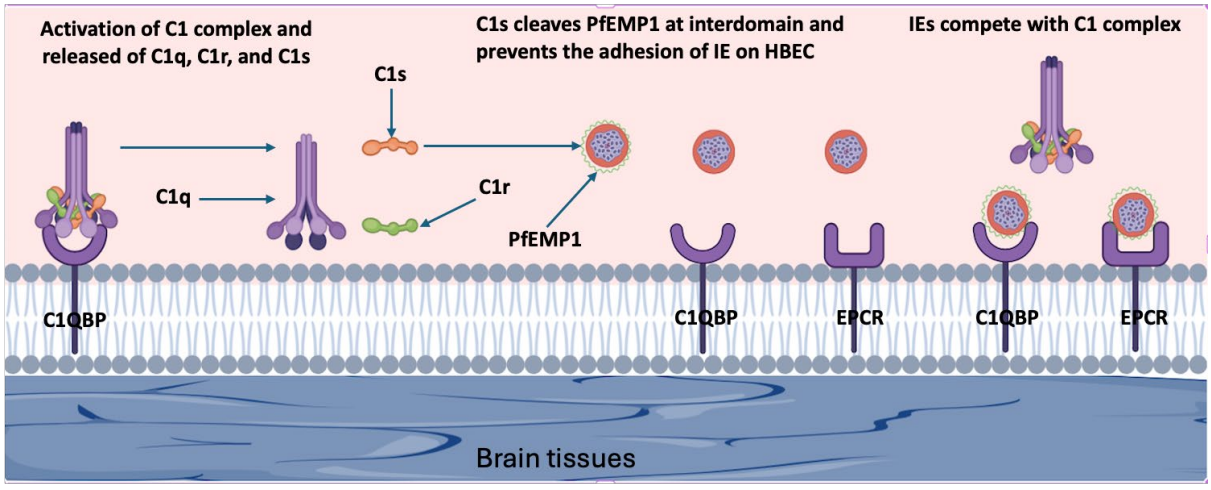


Figure 5-1: Proposed mechanism of C1 complex activation, released of C1s and PfEMP1 cleavage. gC1q can bind to C1QBP, even when it is associated with C1r-C1s complex. gC1q reacts with C1QBP and activates the classical pathway of the complement system. C1s cleaves PfEMP1 at interdomain and abolish the interaction between IE and EPCR, CD36 (Azasi et al., 2021). To ensure its survival and transmission IE develop the ability to interact with C1QBP. The diagram is designed with BioRender.com.

Previous work has suggested that C1s a serine protease of the complement system cleaved PfEMP1 at interdomain between DBLγ and DBLδ (Azasi et al., 2021). This disruption of PfEMP1 abolished the interaction between IE and HBECs receptors like EPCR and CD36 and eventually facilitates IE clearance by the spleen (Buffet et al., 2011). Therefore, the interaction between IT4VAR19-IE and C1QBP (Figure 4-3) may be a strategy for IE to prevent the activation of the complement system and promote IE sequestration via EPCR. In addition, postmortem study from Malawian children with CM showed that cerebral fibrin clots and loss of EPCR colocalised with sequestered IE (Moxon et al., 2013). My hCMEC/D3 IFA data supports the downregulation of EPCR after cell activation with TNF-α (Figure 3-5). These results suggest that EPCR alone cannot mediate the occurrence of CM. Hence, to ensure its survival and transmission IEs have developed the ability to interact with additional receptor such as C1QBP (Figure 4-3). This strategy has been proposed by other authors (Azasi et al., 2018; Heddini et al., 2001; Yipp et al., 2000) and deserved more attention to gain comprehensive understanding of CM pathogenesis.

Host receptor polymorphisms in malaria endemic population play an important role in IE cytoadhesion. They can increase or decrease the susceptibility of malaria endemic population to SM and CM. Point mutations in the haemoglobin gene within malaria endemic population (e.g., HbC, and HbAS) have been associated with SM protection due to the abnormal exhibition of PfEMP1 on the surface of IE (Fairhurst et al., 2012; Petersen et al., 2021; Sanchez et al., 2019) and to form rosettes (Opi et al., 2014). A larger well-designed genome wide association study (GWAS) has identified an association between sickle haemoglobin (HbAS) in the host and three regions of the parasite genome (Band et al., 2022). To date no study has identified a polymorphism in C1QBP and malaria protection or susceptibility. In general, C1QBP is highly conserved in diverse species (Guo et al., 1997).

5.4. Limitations

The study has some limitations, the culture of monolayer hCMEC/D3 and other human endothelial cells (HBEC-5i, primary HBMEC, HPMEC, and HDMEC) are used to understand the pathophysiology of CM in static adhesion (Avril et al., 2013; Avril et al., 2012; Claessens et al., 2012; Jambou et al., 2010; Zougbede et al., 2011). However, these *in vitro* cultured cells do not truly resemble the intricate cellular interactions between different cell types and extracellular matrices of an *in vivo* endothelium. To address these limitations, other researchers

have examined IE cytoadhesion under dynamic flow conditions that simulate microvasculature circulation *in vivo* (Bernabeu et al., 2019). Other groups have also studied cocultures of endothelial cells with pericytes or astrocytes (Liu et al., 2018; Storm et al., 2020), and cerebral organoids (Adams & Jensen, 2022; Adams et al., 2021). The goal is to reproduce as much as possible the complexity of multicellular *in vivo* endothelial tissues and better characterise the pathophysiology of CM.

Another limitation in my study, I have used IFA to generate images, but I did not quantify the expression intensity of endothelial receptors in resting and activated conditions with flow cytometry. It was difficult to appreciate the change of brightness for some receptors between resting and activated hCMEC/D3 cells. It is important to combine IFA and flow cytometry to better characterise surface receptor staining expression. However, surface receptor staining intensity can also be quantified from IFA images using image J (Helmbrecht et al., 2023).

Cytoadhesion data generated in my study are exclusively based on laboratory adapted parasite lines. The small number of parasite lines investigated do not facilitate the detection of difference between parasite strains adhesion and generalise my findings. Particularly the lack of comparison of cytoadhesion between field isolates from patients experiencing UM and CM. Most data about cytoadhesion studies came from laboratory adapted lines that could be used as positive control for study of cytoadhesion with clinical isolates to obtain a complete picture of IE cytoadhesion mechanism.

I have used a small sample size of n=3 independents experiment to analyse my data using either ANOVA or t-test after Log-transformation. I suggest the use of mixed effects models, to increase the sample size of each group to be compared. This method considers each technical replicate as an individual observartion.

5.5. Future work

The purpose of my work is to generate hypotheses that will have to be validated in humans. An important experiment to be performed in the future is to assess whether C1QBP is present *in vivo* on human brain endothelial cells through postmortem investigations. This will emphasize

our findings and lead to a better characterisation of IE sequestration and its interaction with the complement system.

Another line of study would be to identify genetic polymorphisms in C1QBP because IE receptors have been suggested in the resistance to severe malaria (Rowe et al., 2009). Therefore, a polymorphism in the protein C1QBP that conferred a resistance to IE sequestration might protect against CM. However, a recent genome wide association study did not find a polymorphism in candidate endothelial receptors associated with SM in cytoadhesion (Band et al., 2022).

Data presented here are focused on laboratory adapted parasite lines, therefore comparison of C1QBP binding between field isolates from patients affected by UM and CM is of high priority. To determine whether C1QBP binding is associated with an increased risk of SM and CM. In evolutionary prospective, the pressure placed upon malaria parasites by antimalarial drugs, vaccine, and host immunity may change the parasite biology and sequestration receptors. My data suggest multiple adhesion receptors instead of a major receptor (Figure 5-1).

My spot binding data also suggests that HB3VAR03-IE binds only to CD36 (Figure 4-4). However, this receptor is not present on HBECs, therefore other receptors must be responsible for its adhesion to hCMEC/D3. The binding to CD36 is likely due to IEs expressing other PfEMP1 variants, but not the ones expressing the DC13 PfEMP1 HB3VAR03. Therefore, it is crucial to search for the plausible receptor of the native HB3VAR03-IE expressing PfEMP1 DC13.

I found also that PfKE08/9605R+ exhibited an unexpected dual binding phenotype. PfKE08/9605R+ expressed a DC8-like PfEMP1, which contains a DBLβ12 predicted to interact with C1QBP and not ICAM1. However, I observed that 9605R+ IEs bind to EPCR and ICAM1, but the binding mechanism used by 9605R+ IEs to interact with ICAM1 is unknown. There is a need to investigate for that mechanism.

5.6. Conclusion

The general aim of this thesis was to investigate whether C1QBP is a major endothelial cell receptor for IE cytoadhesion. To achieve this aim, hCMEC/D3 was used as a model for IE cytoadhesion and four IEs expressing (IT4VAR19, HB3VAR03, 9197VAR27, and PfKE08/9605R+) were successfully panned for cytoadherence to hCMEC/D3 and rosetting. This selection has led to a significant increase in the expression of a dominant PfEMP1 variant, binding to hCMEC/D3, and rosetting frequency.

The endothelial nature of hCMEC/D3 was authenticated with specific marker for endothelial cells before the investigation of C1QBP and other candidate receptors cellular localisation. Unexpectedly, C1QBP was not constitutively expressed by hCMEC/D3 in resting and activated conditions. However, C1QBP can become associated to the surface membrane of hCMEC/D3 when it is preincubated with soluble C1QBP or normal human plasma (containing soluble C1QBP). I observed the same result with other immortalised HBEC (HBEC-5i) but not the primary HBEC (HBMEC, HPMEC, and HDMEC). All primary HBEC cells investigated natively expressed C1QBP in both resting and activated conditions.

To assess the ability of C1QBP to mediate IE cytoadhesion as the major host endothelial receptor. I found that hCMEC/D3-selected IE (IT4VAR19) bind significantly to recombinant C1QBP but in hCMEC/D3 inhibition assay, antibody to human C1QBP and recombinant C1QBP do not block the interaction between IE and hCMEC/D3. Therefore, C1QBP is not the major endothelial receptor for IE cytoadhesion.

Here I showed that hCMEC/D3 supports IE selection, expression of a predominant PfEMP1 variant, binding to IE, expression of endothelial receptors for IE cytoadhesion, and HBEC inhibition assay. I can argue that hCMEC/D3 cell line is a suitable model for IE cytoadhesion, however the lack of constitutive C1QBP surface expression show that it differs from primary HBEC cells. C1QBP is not the major endothelial cell receptor for IE cytoadhesion but it may act in association with another receptor such as EPCR.

6. Appendix

Table 6-1: Summary of value calculated from absorbance (450 nm) raw data

Well	Content	Raw Data (450)	Raw data Average	Corrected	Conc ng/ml
A01	Blank A1	0.131	0.12525	0	0
A02	Blank A2	0.126			
A03	Blank A3	0.124			
A04	Blank A4	0.12			
A05	AH012 dilution 1/4	0.394	0.4055	0.28025	
A06	AH012 dilution 1/4	0.417			
A07	AH032 dilution 1/4	0.389	0.402	0.27675	
A08	AH032 dilution 1/4	0.415			
B01	Standard 1/64	0.229	0.2405	0.11525	0.156
B02	Standard 1/64	0.252			
B03	Sample (-70) dilution 1/4	1.225	1.169	1.04375	
B04	Sample (-70) dilution 1/4	1.113			
B05	AH012 dilution 1/2	0.471	0.482	0.35675	
B06	AH012 dilution 1/2	0.493			
B07	AH032 dilution 1/2	0.449	0.472	0.34675	
B08	AH032 dilution 1/2	0.495			
C01	Standard 1/32	0.332	0.3445	0.21925	0.312
C02	Standard 1/32	0.357			

C03	Sample (-70) dilution 1/2	2.018	2.019	1.89375	
C04	Sample (-70) dilution 1/2	2.02			
C05	AH011 dilution 1/4	0.485	0.49	0.36475	
C06	AH011 dilution 1/4	0.495			
C07	AH030 dilution 1/4	1.66	1.6765	1.55125	
C08	AH030 dilution 1/4	1.693			
D01	Standard 1/16	0.525	0.523	0.39775	0.625
D02	Standard 1/16	0.521			
D03	Sample (4) dilution 1/4	1.043	1.018	0.89275	
D04	Sample (4) dilution 1/4	0.993			
D05	AH011 dilution 1/2	0.545	0.5295	0.40425	
D06	AH011 dilution 1/2	0.514			
D07	AH030 dilution 1/2	2.373	2.325	2.19975	
D08	AH030 dilution 1/2	2.277			
E01	Standard 1/8	0.778	0.7655	0.64025	1.125
E02	Standard 1/8	0.753			
E03	Sample (4) dilution 1/2	1.879	1.9105	1.78525	
E04	Sample (4) dilution 1/2	1.942			
E05	AH009 dilution 1/4	0.407	0.4065	0.28125	
E06	AH009 dilution 1/4	0.406			

E07	AH027 dilution 1/4	0.368	0.3685	0.24325	
E08	AH027 dilution 1/4	0.369			
F01	Standard 1/4	1.18	1.139	1.01375	2.5
F02	Standard 1/4	1.098			
F03	Sample fresh dilution 1/4	2.923	2.913	2.78775	
F04	Sample fresh dilution 1/4	2.903			
F05	AH009 dilution 1/2	0.454	0.4625	0.33725	
F06	AH009 dilution 1/2	0.471			
F07	AH027 dilution 1/2	0.457	0.46	0.33475	
F08	AH027 dilution 1/2	0.463			
G01	Standard 1/2	1.559	1.523	1.39775	5
G02	Standard 1/2	1.487			
G03	Sample fresh dilution 1/2	3.044	3.003	2.87775	
G04	Sample fresh dilution 1/2	2.962			
G05	AH006 dilution 1/4	0.552	0.536	0.41075	
G06	AH006 dilution 1/4	0.52			
G07	AH025 dilution 1/4	0.386	0.3865	0.26125	
G08	AH025 dilution 1/4	0.387			
H01	Standard undiluted	1.833	1.8385	1.71325	10
H02	Standard undiluted	1.844			

H03	Sample fresh undiluted	3.077	3.059	2.93375	
H04	Sample fresh undiluted	3.041			
H05	AH006 dilution 1/2	0.63	0.6175	0.449225	
H06	AH006 dilution 1/2	0.605			
H07	AH025 dilution 1/2	0.477	0.4765	0.35125	
H08	AH025 dilution 1/2	0.476			

NB: Positive control dilution values are colored in blue; **Conc:** Concentration

To investigate the endothelial nature of primary HPMEC, the cells were fixed, permeabilised and labelled with rabbit polyclonal antibody anti-human vWF. As expected, resting HPMEC cells showed the presence of positive intracytoplasmic granule staining compared to their negative controls, who exhibited only background staining (Figure 6-1, rows 1, 2, and 3 from the top). These results validate the endothelial nature of primary HPMEC.

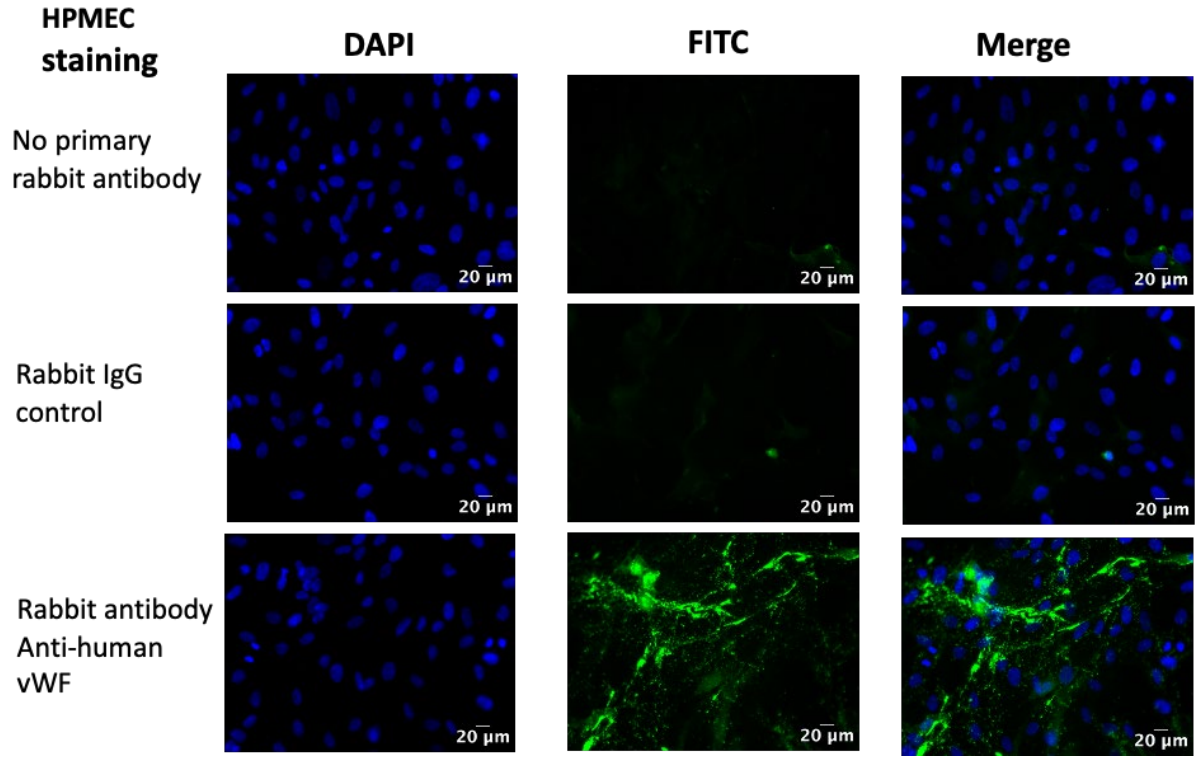


Figure 6-1: Authentication of primary HPMEC as human endothelial cell nature. Resting HPMEC cells were fixed with 1% paraformaldehyde, permeabilised with 0.1% Triton X-100, and blocked with 5% goat serum and then stained with rabbit polyclonal antibody anti-human vWF. Immunofluorescence was detected by an Alexa fluor 488 goat anti-rabbit IgG and visualised with FITC filter (green), counterstained nuclei with DAPI and visualised with the corresponding filter (Blue). Independent experiments were conducted at least twice for this marker and images showed are representatives. All pictures were taken at x400 magnification with a scale bar of 20μm.

I stained fixed primary HPMEC in resting and activated conditions with monoclonal antibody anti-human ICAM1 as a positive control for human endothelial cells. HPMEC cells did display surface expression of host ICAM1 (Figure 6-2, rows 1 and 2 from the bottom) in both conditions. The activation of primary HPMEC cells with TNF-a virtually increases the surface membrane expression of host ICAM1 (Figure 6-2, row 1 from the bottom).

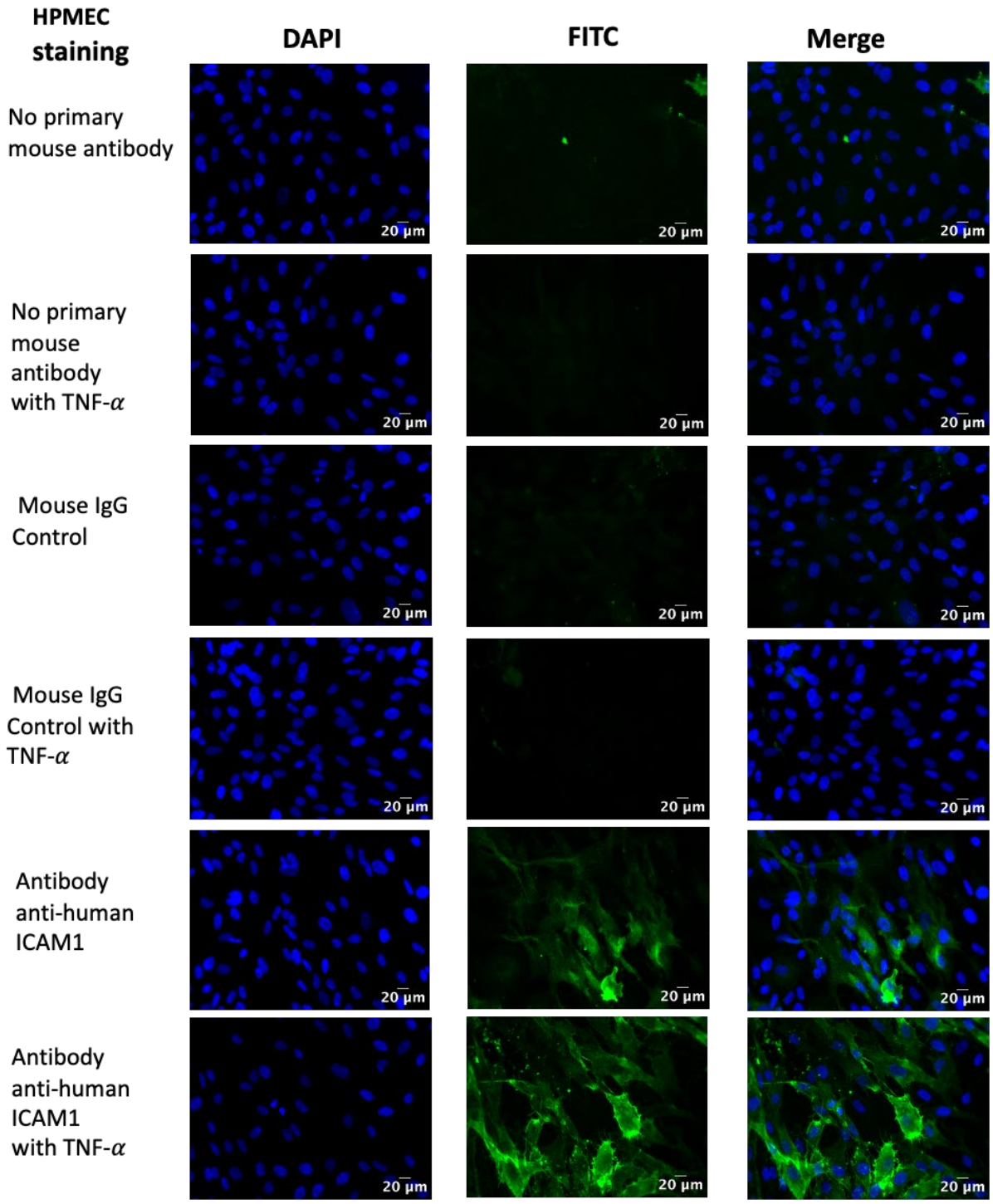


Figure 6-2: Staining of primary HPMEC cells with mouse monoclonal antibody anti-human ICAM1. Resting and activated primary HPMEC cells were fixed with 1% paraformaldehyde, blocked with 5% goat serum and then stained with mouse monoclonal antibody anti-human ICAM1. Immunofluorescence was detected by an Alexa fluor 488 goat anti-mouse IgG and visualised with FITC filter (green), counterstained nuclei with DAPI and visualised with the corresponding filter (Blue). Independent experiments were conducted at least twice for this receptor and images showed are representatives. All pictures were taken at x400 magnification with a scale bar of 20µm.

I assessed the expression of host C1QBP by primary HPMEC. Fixed primary HPMEC cells in resting and activated conditions constitutively exhibit surface expression of host C1QBP compared to their negative controls, who display background level staining (Figure 6-3, rows 1 and 2 from the bottom and rows 1, 2, 3 and 4 from the top). C1QBP is constitutively expressed by primary HPMEC in both conditions.

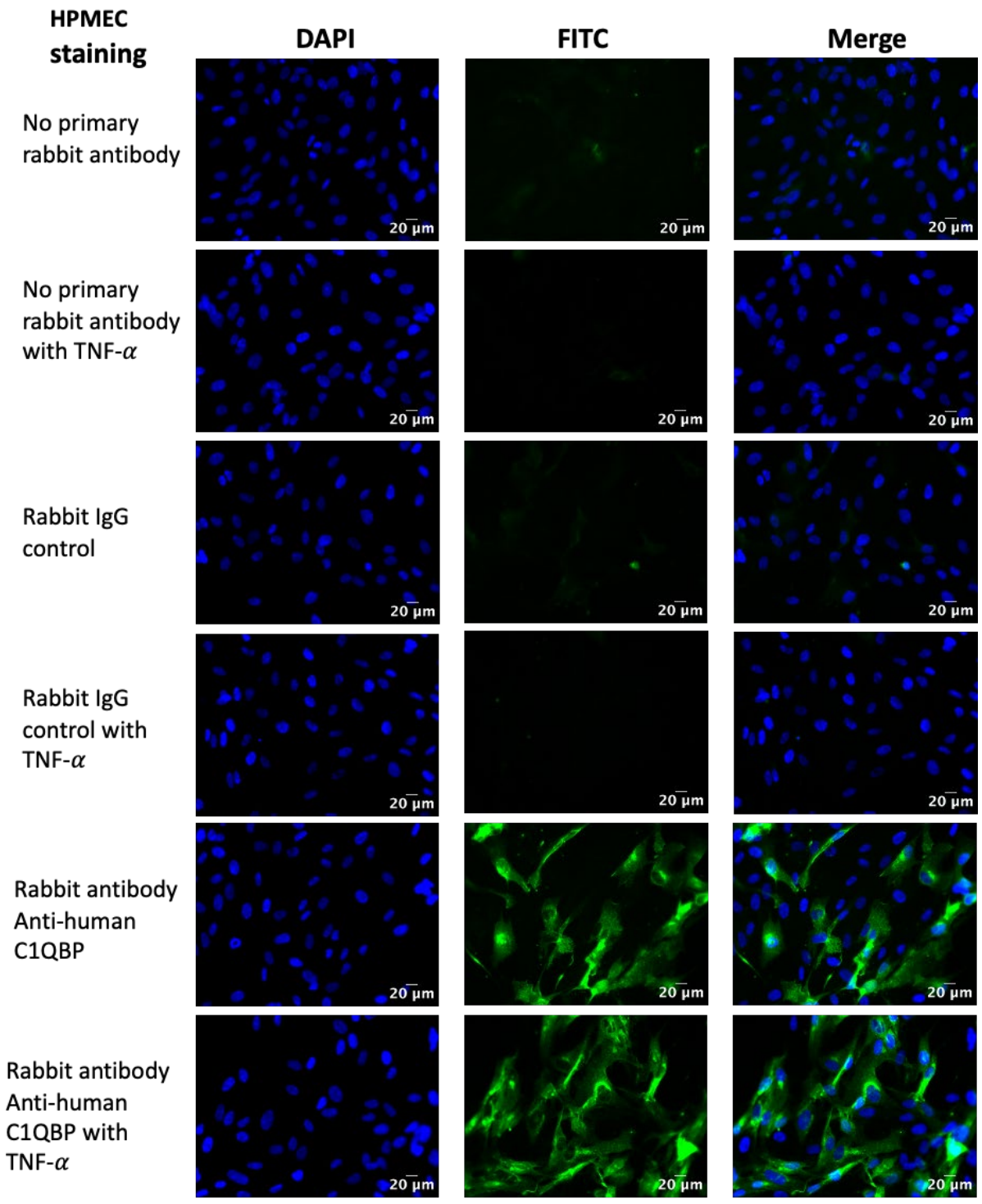


Figure 6-3: Staining of ptimary HPMEC with rabbit polyclonal antibody anti-human C1QBP. Resting and activated primary HBMEC cells were fixed with 1% paraformaldehyde, blocked with 5% goat serum and then stained with rabbit polyclonal antibody anti-human C1QBP. Immunofluorescence was detected by an Alexa fluor 488 goat anti-rabbit IgG and visualised with FITC filter (green), counterstained nuclei with DAPI and visualised with the corresponding filter (Blue). Independent experiments were conducted at least twice for this receptor and images showed are representatives. All pictures were taken at x400 magnification with a scale bar of 20μm.

I tested whether the preincubation of primary HPMEC cells with soluble C1QBP potentiates the expression of C1QBP in IFA. HPMEC shows surface expression of C1QBP in resting and activated conditions (Figure 6-4, rows 1 and 2 from the bottom).

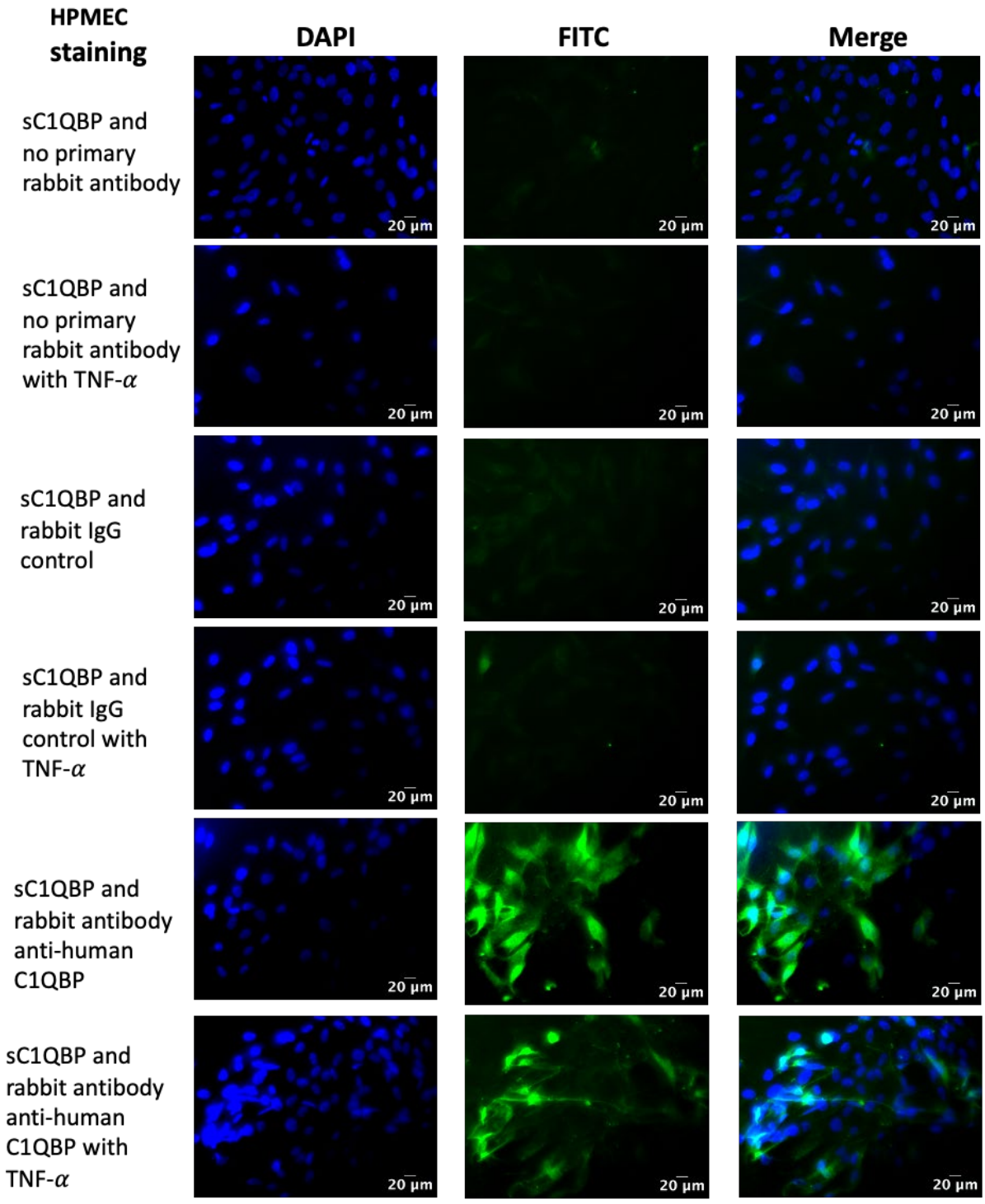


Figure 6-4: Preincubation of primary HPMEC with soluble C1QBP and staining with rabbit polyclonal antibody anti-human C1QBP. Resting and activated primary HPMEC cells were preincubated with soluble C1QBP, and cells were fixed with 1% paraformaldehyde, blocked with 5% goat serum, and stained with rabbit polyclonal antibody anti-human C1QBP. Immunofluorescence was detected by a secondary Alexa fluor 488 goat anti-rabbit IgG conjugated and read with FITC filter (green) and counterstained the cell nuclei with DAPI and read with DAPI filter (Blue). Independent experiments were conducted at least twice and images showed are representatives. All pictures were taken at x400 magnification with a scale bar of 20µm.

To evaluate the endothelial nature of primary HDMEC, the cells were fixed, permeabilised and stained with rabbit polyclonal antibody anti-human vWF. As expected, resting HDMEC cells showed the presence of positive intracytoplasmic granule staining distinctly to their negative controls, who displayed only background staining (Figure 6-5, rows 1, 2, and 3 from the top). These results validate the endothelial nature of primary HDMEC.

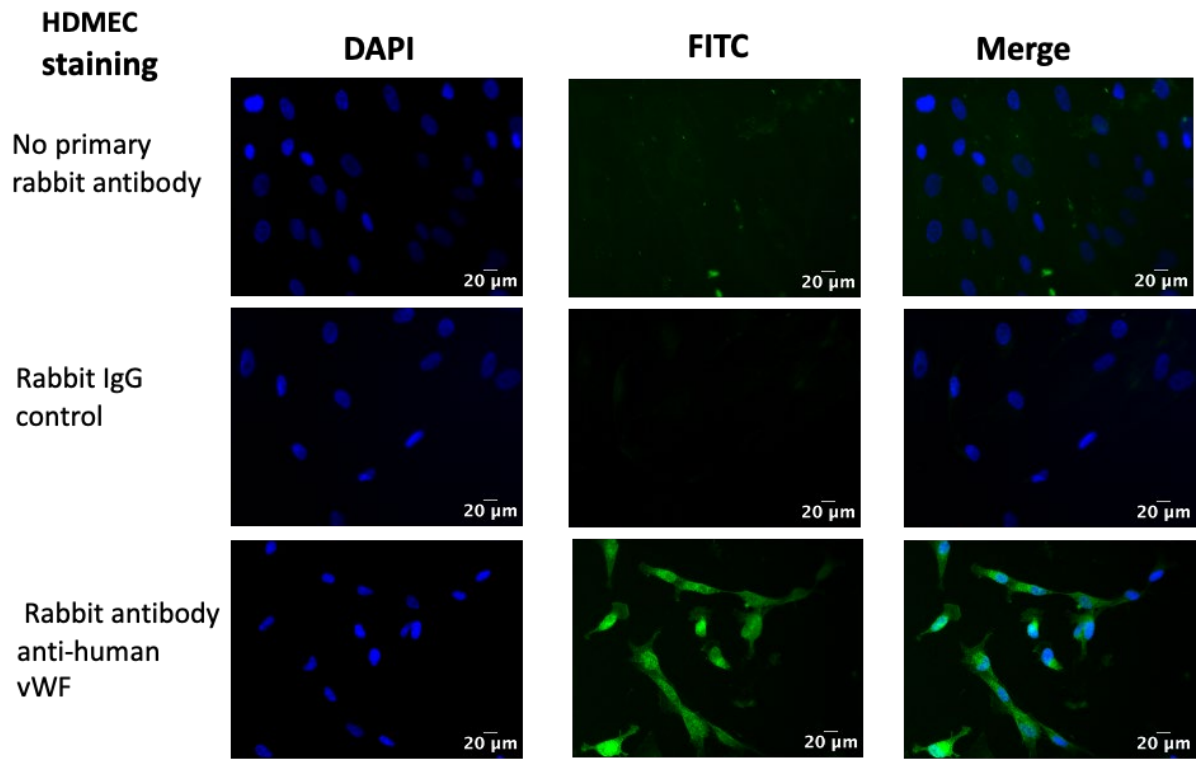


Figure 6-5: Authentication of primary HDMEC as human endothelial cell nature. Resting HDMEC cells were fixed with 1% paraformaldehyde, permeabilised with 0.1% Triton X-100, and blocked with 5% goat serum and then stained with rabbit polyclonal antibody anti-human vWF. Immunofluorescence was detected by an Alexa fluor 488 goat anti-rabbit IgG and visualised with FITC filter (green), counterstained nuclei with DAPI and visualised with the corresponding filter (Blue). Independent experiments were conducted at least twice for this marker and images showed are representatives. All pictures were taken at x400 magnification with a scale bar of 20µm.

I evaluated the expression of host C1QBP by primary HDMEC. Fixed primary HDMEC cells in resting and activated conditions constitutively show surface expression of host C1QBP compared to their negative controls, who display background level staining (Figure 6-6, rows 1 and 2 from the bottom and rows 1, 2, 3 and 4 from the top).

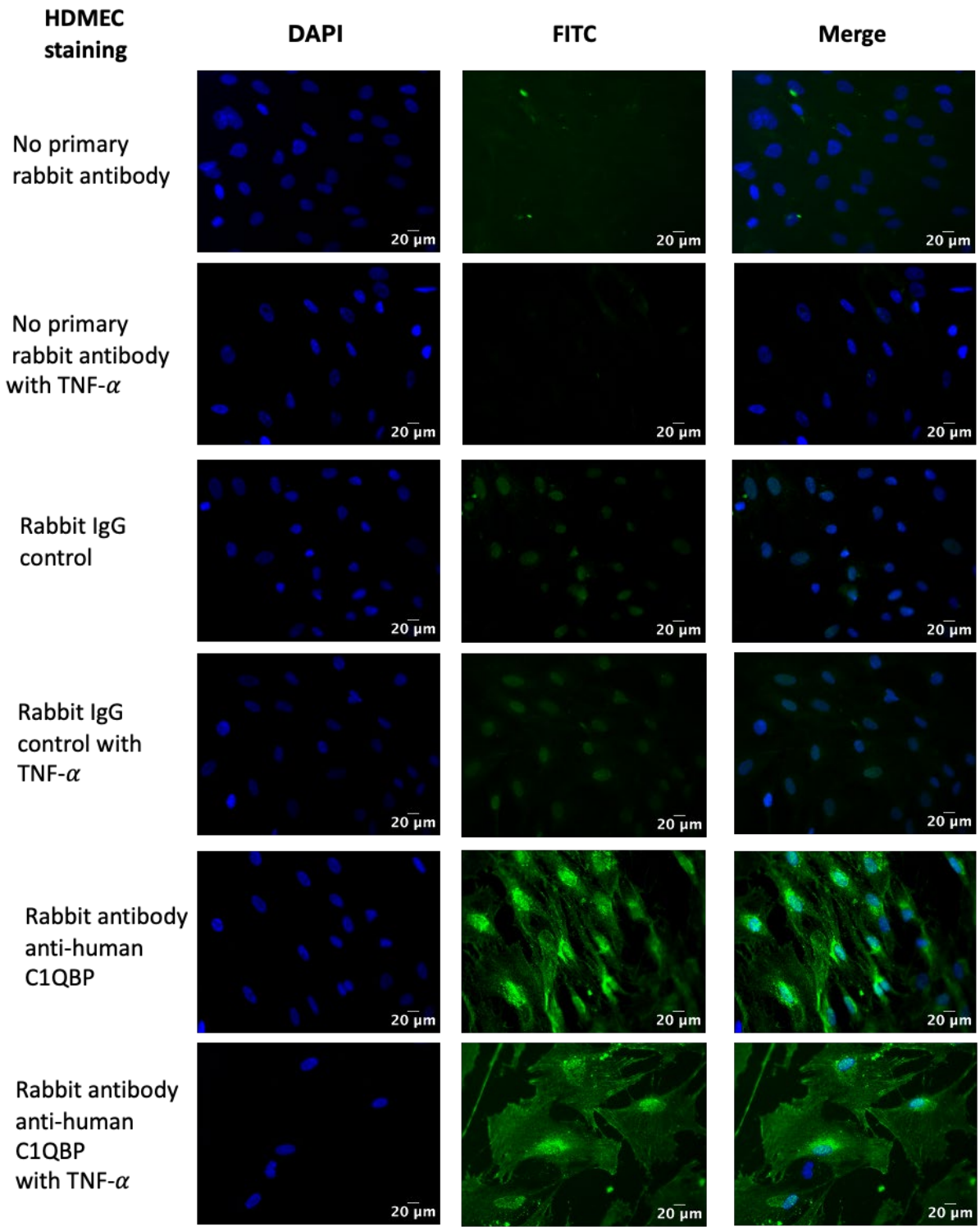


Figure 6-6: Staining of primary HDMEC with rabbit polyclonal antibody anti-human C1QBP. Resting and activated primary HDMEC cells were fixed with 1% paraformaldehyde, blocked with 5% goat serum and then stained with rabbit polyclonal antibody anti-human C1QBP. Immunofluorescence was detected by an Alexa fluor 488 goat anti-rabbit IgG and visualised with FITC filter (green), counterstained nuclei with DAPI and visualised with the corresponding filter (Blue). Independent experiments were conducted at least twice for this receptor and images shown are representatives. All pictures were taken at x400 magnification with a scale bar of 20µm.

I tested whether the preincubation of primary HDMEC cells with soluble C1QBP improves the expression of C1QBP in IFA. C1QBP is still present on the surface of resting and activated HDMEC (Figure 6-7, rows 1 and 2 from the bottom and rows 1, 2, 3 and 4 from the top).

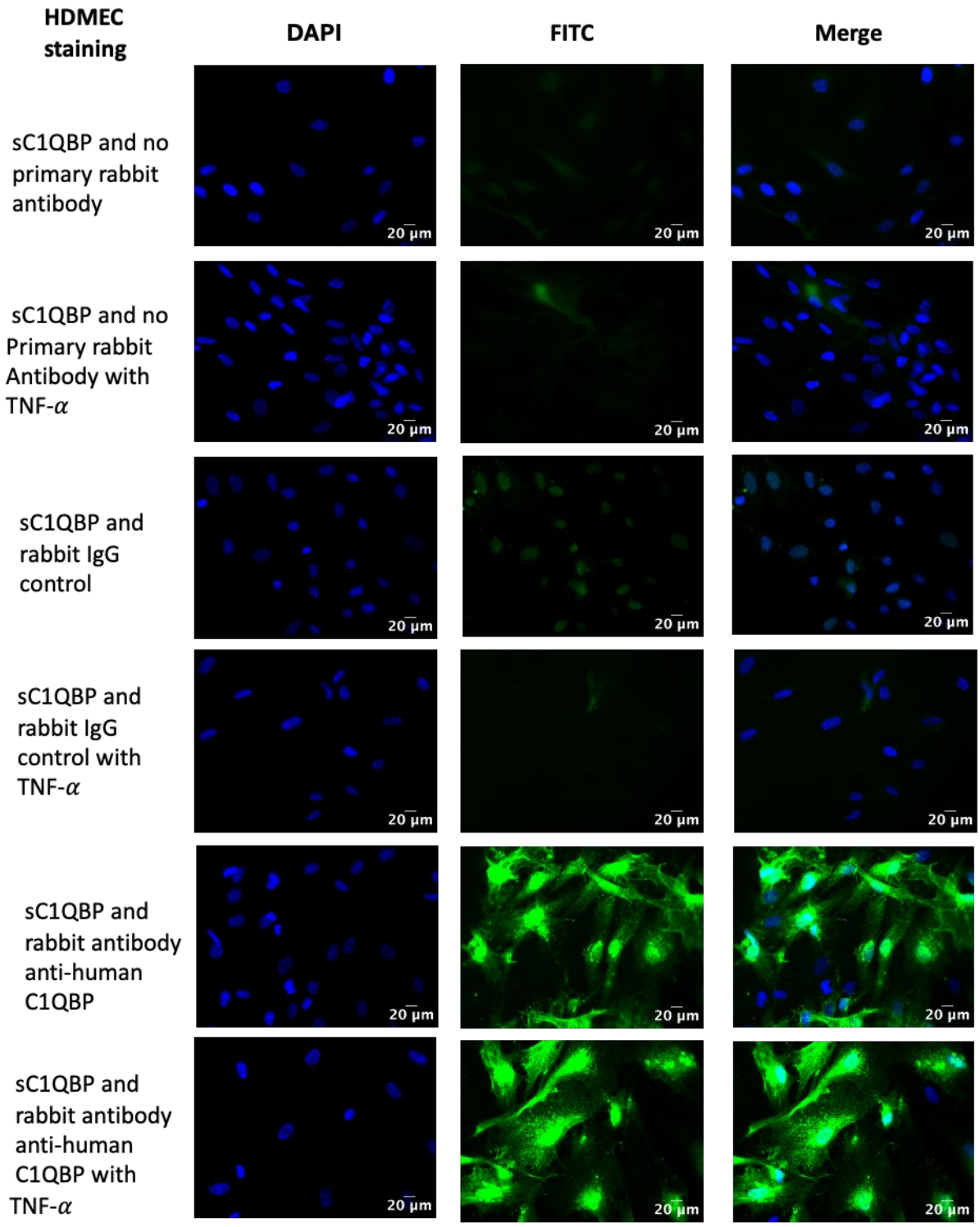


Figure 6-7: Preincubation of primary HDMEC with soluble C1QBP and stainong with rabbit polyclonal anti-human. Resting and activated primary HDMEC cells were preincubated with soluble C1QBP and then, cells were fixed with 1% paraformaldehyde, blocked with 5% goat serum, and stained with rabbit polyclonal antibody anti-human C1QBP. Immunofluorescence was detected by a secondary Alexa fluor 488 goat anti-rabbit IgG conjugated and read with FITC filter (green) and counterstained the cell nuclei with DAPI and read with DAPI filter (Blue). Independent experiments were conducted at least twice and images showed are representatives. All pictures were taken at x400 magnification with a scale bar of 20µm.

7. References

Adams, Y., & Jensen, A. R. (2022). Cerebral malaria - modelling interactions at the blood-brain barrier in vitro. *Dis Model Mech*, *15*(7). https://doi.org/10.1242/dmm.049410

Adams, Y., Kuhnrae, P., Higgins, M. K., Ghumra, A., & 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. *Infect Immun*, *82*(3), 949-959. https://doi.org/10.1128/IAI.01233-13

Adams, Y., Olsen, R. W., Bengtsson, A., Dalgaard, N., Zdioruk, M., Satpathi, S., Behera, P. K., Sahu, P. K., Lawler, S. E., Qvortrup, K., Wassmer, S. C., & Jensen, A. T. R. (2021). Plasmodium falciparum erythrocyte membrane protein 1 variants induce cell swelling and disrupt the blood-brain barrier in cerebral malaria. *J Exp Med*, *218*(3). https://doi.org/10.1084/jem.20201266

Andrews, B. S., Shadforth, M., Cunningham, P., & Davis, J. S. t. (1981). Demonstration of a C1q receptor on the surface of human endothelial cells. *J Immunol*, *127*(3), 1075-1080.

Arman, M., Raza, A., Tempest, L. J., Lyke, K. E., Thera, M. A., Koné, A., Plowe, C. V., Doumbo, O. K., & Rowe, J. A. (2007). Platelet-mediated clumping of Plasmodium falciparum infected erythrocytes is associated with high parasitemia but not severe clinical manifestations of malaria in African children. *Am J Trop Med Hyg*, *77*(5), 943-946.

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., . . . Tracking Resistance to Artemisinin, C. (2014). Spread of artemisinin resistance in Plasmodium falciparum malaria. *N Engl J Med*, *371*(5), 411-423. https://doi.org/10.1056/NEJMoa1314981

Avril, M., Benjamin, M., Dols, M. M., & Smith, J. D. (2019). Interplay of Plasmodium falciparum and thrombin in brain endothelial barrier disruption. *Sci Rep*, *9*(1), 13142. https://doi.org/10.1038/s41598-019-49530-1

Avril, M., Bernabeu, M., Benjamin, M., Brazier, A. J., & 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). https://doi.org/10.1128/mBio.00615-16

Avril, M., Brazier, A. J., Melcher, M., Sampath, S., & Smith, J. D. (2013). DC8 and DC13 var genes associated with severe malaria bind avidly to diverse endothelial cells. *PLoS Pathog*, *9*(6), e1003430. https://doi.org/10.1371/journal.ppat.1003430

Avril, M., Tripathi, A. K., Brazier, A. J., Andisi, C., Janes, J. H., Soma, V. L., Sullivan, D. J., Jr., Bull, P. C., Stins, M. F., & Smith, J. D. (2012). A restricted subset of var genes mediates adherence of Plasmodium falciparum-infected erythrocytes to brain endothelial cells. *Proc Natl Acad Sci U S A*, *109*(26), E1782-1790. https://doi.org/10.1073/pnas.1120534109

Awandare, G. A., Kempaiah, P., Ochiel, D. O., Piazza, P., Keller, C. C., & Perkins, D. J. (2011). Mechanisms of erythropoiesis inhibition by malarial pigment and malaria-induced proinflammatory mediators in an in vitro model. *Am J Hematol*, *86*(2), 155-162. https://doi.org/10.1002/ajh.21933

Azasi, Y., Lindergard, G., Ghumra, A., Mu, J., Miller, L. H., & Rowe, J. A. (2018). Infected erythrocytes expressing DC13 PfEMP1 differ from recombinant proteins in EPCR-binding function. *Proc Natl Acad Sci U S A*, *115*(5), 1063-1068. https://doi.org/10.1073/pnas.1712879115

Azasi, Y., Low, L. M., Just, A. N., Raghavan, S. S. R., Wang, C. W., Valenzuela-Leon, P., Rowe, J. A., Smith, J. D., Lavstsen, T., Turner, L., Calvo, E., & Miller, L. H. (2021). Complement C1s cleaves PfEMP1 at interdomain conserved sites inhibiting Plasmodium falciparum cytoadherence. *Proc Natl Acad Sci U S A*, *118*(22). https://doi.org/10.1073/pnas.2104166118

Bakri, R., Rehan, M., Shamshad, H., & Hafiz, A. (2021). Computational Insights into the Interaction between Cytoadherence Receptor gC1qR and the DBLbeta12 Domain of a Plasmodium falciparum PfEMP1 Ligand. *Life (Basel)*, *11*(9). https://doi.org/10.3390/life11090993

Balikagala, B., Fukuda, N., Ikeda, M., Katuro, O. T., Tachibana, S. I., Yamauchi, M., Opio, W., Emoto, S., Anywar, D. A., Kimura, E., Palacpac, N. M. Q., Odongo-Aginya, E. I., Ogwang, M., Horii, T., & Mita, T. (2021). Evidence of Artemisinin-Resistant Malaria in Africa. *N Engl J Med*, *385*(13), 1163-1171. https://doi.org/10.1056/NEJMoa2101746

Band, G., Leffler, E. M., Jallow, M., Sisay-Joof, F., Ndila, C. M., Macharia, A. W., Hubbart, C., Jeffreys, A. E., Rowlands, K., Nguyen, T., Goncalves, S., Ariani, C. V., Stalker, J., Pearson, R. D., Amato, R., Drury, E., Sirugo, G., d'Alessandro, U., Bojang, K. A., . . . Kwiatkowski, D. P. (2022). Malaria protection due to sickle haemoglobin depends on parasite genotype. *Nature*, *602*(7895), 106-111. https://doi.org/10.1038/s41586-021-04288-3

Barnwell, J. W., Asch, A. S., Nachman, R. L., Yamaya, M., Aikawa, M., & 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. *J Clin Invest*, *84*(3), 765-772. https://doi.org/10.1172/jci114234

Barnwell, J. W., Ockenhouse, C. F., & Knowles, D. M., 2nd. (1985). Monoclonal antibody OKM5 inhibits the in vitro binding of Plasmodium falciparum-infected erythrocytes to monocytes, endothelial, and C32 melanoma cells. *J Immunol*, *135*(5), 3494-3497.

Barrera, V., MacCormick, I. J. C., Czanner, G., Hiscott, P. S., White, V. A., Craig, A. G., Beare, N. A. V., Culshaw, L. H., Zheng, Y., Biddolph, S. C., Milner, D. A., Kamiza, S., Molyneux, M. E., Taylor, T. E., & Harding, S. P. (2018). Neurovascular sequestration in paediatric P. falciparum malaria is visible clinically in the retina. *Elife*, *7*. https://doi.org/10.7554/eLife.32208

Baruch, D. I., Pasloske, B. L., Singh, H. B., Bi, X., Ma, X. C., Feldman, M., Taraschi, T. F., & 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), 77-87. https://doi.org/10.1016/0092-8674(95)90054-3

Berendt, A. R., McDowall, A., Craig, A. G., Bates, P. A., Sternberg, M. J., Marsh, K., Newbold, C. I., & Hogg, N. (1992). The binding site on ICAM-1 for Plasmodium falciparum-infected erythrocytes overlaps, but is distinct from, the LFA-1-binding site. *Cell*, *68*(1), 71-81. https://doi.org/10.1016/0092-8674(92)90207-s

Berendt, A. R., Simmons, D. L., Tansey, J., Newbold, C. I., & Marsh, K. (1989). Intercellular adhesion molecule-1 is an endothelial cell adhesion receptor for Plasmodium falciparum. *Nature*, *341*(6237), 57-59. https://doi.org/10.1038/341057a0

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., & Smith, J. D. (2016). Severe adult malaria is associated with specific PfEMP1 adhesion types and high parasite biomass. *Proc Natl Acad Sci U S A*, *113*(23), E3270-3279. https://doi.org/10.1073/pnas.1524294113

Bernabeu, M., Gunnarsson, C., Vishnyakova, M., Howard, C. C., Nagao, R. J., Avril, M., Taylor, T. E., Seydel, K. B., Zheng, Y., & Smith, J. D. (2019). Binding Heterogeneity

of Plasmodium falciparum to Engineered 3D Brain Microvessels Is Mediated by EPCR and ICAM-1. *mBio*, *10*(3). https://doi.org/10.1128/mBio.00420-19

Bernabeu, M., & Smith, J. D. (2017). EPCR and Malaria Severity: The Center of a Perfect Storm. *Trends Parasitol*, *33*(4), 295-308. https://doi.org/10.1016/j.pt.2016.11.004

Bhasin, V. K., & Trager, W. (1984). Gametocyte-forming and non-gametocyte-forming clones of Plasmodium falciparum. *Am J Trop Med Hyg*, *33*(4), 534-537. https://doi.org/10.4269/ajtmh.1984.33.534

Biswas, A. K., Hafiz, A., Banerjee, B., Kim, K. S., Datta, K., & Chitnis, C. E. (2007). Plasmodium falciparum uses gC1qR/HABP1/p32 as a receptor to bind to vascular endothelium and for platelet-mediated clumping. *PLoS Pathog*, *3*(9), 1271-1280. https://doi.org/10.1371/journal.ppat.0030130

Bond, C., Bednarski, O. J., Datta, D., Namazzi, R., Opoka, R. O., Lima-Cooper, G., Batte, A., Udumula, K., Balasubramani, D., Vasquez, M., Rodriguez, A., Liepmann, C., Bangirana, P., Abreu, M., Schwantes-An, T. H., Zhao, Y., El-Achkar, T. M., Schmidt, N. W., John, C. C., & Conroy, A. L. (2025). Elevated uric acid levels, mortality and cognitive impairment in children with severe malaria. *Nat Med*. https://doi.org/10.1038/s41591-024-03430-8

Bordin, S., Kolb, W. P., & Page, R. C. (1983). C1Q receptors on cultured human gingival fibroblasts: analysis of binding properties. *J Immunol*, *130*(4), 1871-1875.

Bruni, R., & Roizman, B. (1996). Open reading frame P--a herpes simplex virus gene repressed during productive infection encodes a protein that binds a splicing factor and reduces synthesis of viral proteins made from spliced mRNA. *Proc Natl Acad Sci U S A*, *93*(19), 10423-10427. https://doi.org/10.1073/pnas.93.19.10423

Buffet, P. A., Safeukui, I., Deplaine, G., Brousse, V., Prendki, V., Thellier, M., Turner, G. D., & Mercereau-Puijalon, O. (2011). The pathogenesis of Plasmodium falciparum malaria in humans: insights from splenic physiology. *Blood*, *117*(2), 381-392. https://doi.org/10.1182/blood-2010-04-202911

Calis, J. C., Phiri, K. S., Faragher, E. B., Brabin, B. J., Bates, I., Cuevas, L. E., de Haan, R. J., Phiri, A. I., Malange, P., Khoka, M., Hulshof, P. J., van Lieshout, L., Beld, M. G., Teo, Y. Y., Rockett, K. A., Richardson, A., Kwiatkowski, D. P., Molyneux, M. E., & van Hensbroek, M. B. (2016). Severe anemia in Malawian children. *Malawi Med J*, *28*(3), 99-107.

Carlson, J., Helmby, H., Hill, A. V., Brewster, D., Greenwood, B. M., & Wahlgren, M. (1990). Human cerebral malaria: association with erythrocyte rosetting and lack of anti-rosetting antibodies. *Lancet*, *336*(8729), 1457-1460. https://doi.org/10.1016/0140-6736(90)93174-n

Casals-Pascual, C., Kai, O., Cheung, J. O., Williams, S., Lowe, B., Nyanoti, M., Williams, T. N., Maitland, K., Molyneux, M., Newton, C. R., Peshu, N., Watt, S. M., & Roberts, D. J. (2006). Suppression of erythropoiesis in malarial anemia is associated with hemozoin in vitro and in vivo. *Blood*, *108*(8), 2569-2577. https://doi.org/10.1182/blood-2006-05-018697

Ch'ng, J. H., Moll, K., Quintana Mdel, P., Chan, S. C., Masters, E., Moles, E., Liu, J., Eriksson, A. B., & Wahlgren, M. (2016). Rosette-Disrupting Effect of an Anti-Plasmodial Compound for the Potential Treatment of Plasmodium falciparum Malaria Complications. *Sci Rep*, *6*, 29317. https://doi.org/10.1038/srep29317

Cham, G. K., Turner, L., Kurtis, J. D., Mutabingwa, T., Fried, M., Jensen, A. T., Lavstsen, T., Hviid, L., Duffy, P. E., & Theander, T. G. (2010). Hierarchical, domain type-specific acquisition of antibodies to Plasmodium falciparum erythrocyte membrane protein 1 in Tanzanian children. *Infect Immun*, *78*(11), 4653-4659. https://doi.org/10.1128/IAI.00593-10

Cham, G. K., Turner, L., Lusingu, J., Vestergaard, L., Mmbando, B. P., Kurtis, J. D., Jensen, A. T., Salanti, A., Lavstsen, T., & Theander, T. G. (2009). Sequential, ordered acquisition of antibodies to Plasmodium falciparum erythrocyte membrane protein 1 domains. *J Immunol*, *183*(5), 3356-3363. https://doi.org/10.4049/jimmunol.0901331

Cheung, A. L., Krishnan, M., Jaffe, E. A., & Fischetti, V. A. (1991). Fibrinogen acts as a bridging molecule in the adherence of Staphylococcus aureus to cultured human endothelial cells. *J Clin Invest*, *87*(6), 2236-2245. https://doi.org/10.1172/JCI115259

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., & Rowe, J. A. (2012). A subset of group A-like var genes encodes the malaria parasite ligands for binding to human brain endothelial cells. *Proc Natl Acad Sci U S A*, *109*(26), E1772-1781. https://doi.org/10.1073/pnas.1120461109

Claessens, A., & Rowe, J. A. (2012). Selection of Plasmodium falciparum parasites for cytoadhesion to human brain endothelial cells. *J Vis Exp*(59), e3122. https://doi.org/10.3791/3122

Clark, D. J., Bond, C., Andrews, A., Muller, D. J., Sarkisian, A., Opoka, R. O., Idro, R., Bangirana, P., Witten, A., Sausen, N. J., Birbeck, G. L., John, C. C., & Postels, D. G. (2023). Admission Clinical and EEG Features Associated With Mortality and Long-term Neurologic and Cognitive Outcomes in Pediatric Cerebral Malaria. *Neurology*, *101*(13), e1307-e1318. https://doi.org/10.1212/WNL.0000000000207657

Cojean, S., Jafari-Guemouri, S., Le Bras, J., & 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), 163-169. https://doi.org/10.1051/parasite/2008152163

Conroy, A. L., Datta, D., Hoffmann, A., & Wassmer, S. C. (2023). The kidney-brain pathogenic axis in severe falciparum malaria. *Trends Parasitol*, *39*(3), 191-199. https://doi.org/10.1016/j.pt.2023.01.005

Cowman, A. F., Healer, J., Marapana, D., & Marsh, K. (2016). Malaria: Biology and Disease. *Cell*, *167*(3), 610-624. https://doi.org/10.1016/j.cell.2016.07.055

Daha, M. R., Miltenburg, A. M., Hiemstra, P. S., Klar-Mohamad, N., Van Es, L. A., & Van Hinsbergh, V. W. (1988). The complement subcomponent C1q mediates binding of immune complexes and aggregates to endothelial cells in vitro. *Eur J Immunol*, *18*(5), 783-787. https://doi.org/10.1002/eji.1830180519

Davis, S. P., Lee, K., Gillrie, M. R., Roa, L., Amrein, M., & Ho, M. (2013). CD36 recruits alpha(5)beta(1) integrin to promote cytoadherence of P. falciparum-infected erythrocytes. *PLoS Pathog*, *9*(8), e1003590. https://doi.org/10.1371/journal.ppat.1003590

de Souza, J. B., Hafalla, J. C., Riley, E. M., & Couper, K. N. (2010). Cerebral malaria: why experimental murine models are required to understand the pathogenesis of disease. *Parasitology*, *137*(5), 755-772. https://doi.org/10.1017/s0031182009991715

Dedio, J., Jahnen-Dechent, W., Bachmann, M., & Müller-Esterl, W. (1998). The multiligand-binding protein gC1qR, putative C1q receptor, is a mitochondrial protein. *J Immunol*, *160*(7), 3534-3542.

Dedio, J., & Muller-Esterl, W. (1996). Kininogen binding protein p33/gC1qR is localized in the vesicular fraction of endothelial cells. *FEBS Lett*, *399*(3), 255-258. https://doi.org/10.1016/s0014-5793(96)01339-7

Desai, M., ter Kuile, F. O., Nosten, F., McGready, R., Asamoa, K., Brabin, B., & Newman, R. D. (2007). Epidemiology and burden of malaria in pregnancy. *Lancet Infect Dis*, *7*(2), 93-104. https://doi.org/10.1016/S1473-3099(07)70021-X

Diamond, M. S., Staunton, D. E., de Fougerolles, A. R., Stacker, S. A., Garcia-Aguilar, J., Hibbs, M. L., & Springer, T. A. (1990). ICAM-1 (CD54): a counter-receptor for Mac-1 (CD11b/CD18). *J Cell Biol*, *111*(6 Pt 2), 3129-3139. https://doi.org/10.1083/jcb.111.6.3129

Dickler, H. B., & Kunkel, H. G. (1972). Interaction of aggregated -globulin with B lymphocytes. *J Exp Med*, *136*(1), 191-196. https://doi.org/10.1084/jem.136.1.191

Dondorp, A. M., Angus, B. J., Chotivanich, K., Silamut, K., Ruangveerayuth, R., Hardeman, M. R., Kager, P. A., Vreeken, J., & White, N. J. (1999). Red blood cell deformability as a predictor of anemia in severe falciparum malaria. *Am J Trop Med Hyg*, *60*(5), 733-737. https://doi.org/10.4269/ajtmh.1999.60.733

Dondorp, A. M., Angus, B. J., Hardeman, M. R., Chotivanich, K. T., Silamut, K., Ruangveerayuth, R., Kager, P. A., White, N. J., & Vreeken, J. (1997). Prognostic significance of reduced red blood cell deformability in severe falciparum malaria. *Am J Trop Med Hyg*, *57*(5), 507-511. https://doi.org/10.4269/ajtmh.1997.57.507

Dondorp, A. M., Desakorn, V., Pongtavornpinyo, W., Sahassananda, D., Silamut, K., Chotivanich, K., Newton, P. N., Pitisuttithum, P., Smithyman, A. M., White, N. J., & Day, N. P. (2005). Estimation of the total parasite biomass in acute falciparum malaria from plasma PfHRP2. *PLoS Med*, *2*(8), e204. https://doi.org/10.1371/journal.pmed.0020204

Dondorp, A. M., Fanello, C. I., Hendriksen, I. C., Gomes, E., Seni, A., Chhaganlal, K. D., Bojang, K., Olaosebikan, R., Anunobi, N., Maitland, K., Kivaya, E., Agbenyega, T., Nguah, S. B., Evans, J., Gesase, S., Kahabuka, C., Mtove, G., Nadjm, B., Deen, J., . . . group, A. (2010). Artesunate versus quinine in the treatment of severe falciparum malaria in African children (AQUAMAT): an open-label, randomised trial. *Lancet*, *376*(9753), 1647-1657. https://doi.org/10.1016/S0140-6736(10)61924-1

Dondorp, A. M., Lee, S. J., Faiz, M. A., Mishra, S., Price, R., Tjitra, E., Than, M., Htut, Y., Mohanty, S., Yunus, E. B., Rahman, R., Nosten, F., Anstey, N. M., Day, N. P., & White, N. J. (2008). The relationship between age and the manifestations of and mortality associated with severe malaria. *Clin Infect Dis*, *47*(2), 151-157. https://doi.org/10.1086/589287

Dondorp, A. M., Nyanoti, M., Kager, P. A., Mithwani, S., Vreeken, J., & Marsh, K. (2002). The role of reduced red cell deformability in the pathogenesis of severe falciparum malaria and its restoration by blood transfusion. *Trans R Soc Trop Med Hyg*, *96*(3), 282-286. https://doi.org/10.1016/s0035-9203(02)90100-8

Dondorp, A. M., Pongponratn, E., & White, N. J. (2004). Reduced microcirculatory flow in severe falciparum malaria: pathophysiology and electron-microscopic pathology. *Acta Trop*, *89*(3), 309-317. https://doi.org/10.1016/j.actatropica.2003.10.004

Dorovini-Zis, K., Prameya, R., & Bowman, P. D. (1991). Culture and characterization of microvascular endothelial cells derived from human brain. *Lab Invest*, *64*(3), 425-436.

Dorovini-Zis, K., Schmidt, K., Huynh, H., Fu, W., Whitten, R. O., Milner, D., Kamiza, S., Molyneux, M., & Taylor, T. E. (2011). The neuropathology of fatal cerebral malaria in malawian children. *Am J Pathol*, *178*(5), 2146-2158. https://doi.org/10.1016/j.ajpath.2011.01.016

Doumbo, O. K., Thera, M. A., Kone, A. K., Raza, A., Tempest, L. J., Lyke, K. E., Plowe, C. V., & Rowe, J. A. (2009). High levels of Plasmodium falciparum rosetting in all clinical forms of severe malaria in African children. *Am J Trop Med Hyg*, *81*(6), 987-993. https://doi.org/10.4269/ajtmh.2009.09-0406

Duffy, F., Bernabeu, M., Babar, P. H., Kessler, A., Wang, C. W., Vaz, M., Chery, L., Mandala, W. L., Rogerson, S. J., Taylor, T. E., Seydel, K. B., Lavstsen, T., Gomes, E., Kim, K., Lusingu, J., Rathod, P. K., Aitchison, J. D., & Smith, J. D. (2019). Meta-analysis of

Plasmodium falciparum var Signatures Contributing to Severe Malaria in African Children and Indian Adults. *mBio*, *10*(2). https://doi.org/10.1128/mBio.00217-19

Eggleton, P., Ghebrehiwet, B., Sastry, K. N., Coburn, J. P., Zaner, K. S., Reid, K. B., & Tauber, A. I. (1995). Identification of a gC1q-binding protein (gC1q-R) on the surface of human neutrophils. Subcellular localization and binding properties in comparison with the cC1q-R. *J Clin Invest*, *95*(4), 1569-1578. https://doi.org/10.1172/jci117830

Fairhurst, R. M., Baruch, D. I., Brittain, N. J., Ostera, G. R., Wallach, J. S., Hoang, H. L., Hayton, K., Guindo, A., Makobongo, M. O., Schwartz, O. M., Tounkara, A., Doumbo, O. K., Diallo, D. A., Fujioka, H., Ho, M., & Wellems, T. E. (2005). Abnormal display of PfEMP-1 on erythrocytes carrying haemoglobin C may protect against malaria. *Nature*, *435*(7045), 1117-1121. https://doi.org/10.1038/nature03631

Fairhurst, R. M., Bess, C. D., & Krause, M. A. (2012). Abnormal PfEMP1/knob display on Plasmodium falciparum-infected erythrocytes containing hemoglobin variants: fresh insights into malaria pathogenesis and protection. *Microbes Infect*, *14*(10), 851-862. https://doi.org/10.1016/j.micinf.2012.05.006

Febbraio, M., Hajjar, D. P., & Silverstein, R. L. (2001). CD36: a class B scavenger receptor involved in angiogenesis, atherosclerosis, inflammation, and lipid metabolism. *Journal of Clinical Investigation*, *108*(6), 785-791. https://doi.org/10.1172/jci14006

Fogal, V., Richardson, A. D., Karmali, P. P., Scheffler, I. E., Smith, J. W., & Ruoslahti, E. (2010). Mitochondrial p32 protein is a critical regulator of tumor metabolism via maintenance of oxidative phosphorylation. *Mol Cell Biol*, *30*(6), 1303-1318. https://doi.org/10.1128/mcb.01101-09

Francischetti, I. M., Seydel, K. B., Monteiro, R. Q., Whitten, R. O., Erexson, C. R., Noronha, A. L., Ostera, G. R., Kamiza, S. B., Molyneux, M. E., Ward, J. M., & Taylor, T. E. (2007). Plasmodium falciparum-infected erythrocytes induce tissue factor expression in endothelial cells and support the assembly of multimolecular coagulation complexes. *J Thromb Haemost*, *5*(1), 155-165. https://doi.org/10.1111/j.1538-7836.2006.02232.x

Fried, M., Nosten, F., Brockman, A., Brabin, B. J., & Duffy, P. E. (1998). Maternal antibodies block malaria. *Nature*, *395*(6705), 851-852. https://doi.org/10.1038/27570

Gamain, B., Chene, A., Viebig, N. K., Tuikue Ndam, N., & Nielsen, M. A. (2021). Progress and Insights Toward an Effective Placental Malaria Vaccine. *Front Immunol*, *12*, 634508. https://doi.org/10.3389/fimmu.2021.634508

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., . . . Barrell, B. (2002). Genome sequence of the human malaria parasite Plasmodium falciparum. *Nature*, *419*(6906), 498-511. https://doi.org/10.1038/nature01097

Ghebrehiwet, B., Geisbrecht, B. V., Xu, X., Savitt, A. G., & Peerschke, E. I. B. (2019). The C1q Receptors: Focus on gC1qR/p33 (C1qBP, p32, HABP-1)(1). *Semin Immunol*, *45*, 101338. https://doi.org/10.1016/j.smim.2019.101338

Ghebrehiwet, B., Ji, Y., Valentino, A., Pednekar, L., Ramadass, M., Habiel, D., Kew, R. R., Hosszu, K. H., Galanakis, D. K., Kishore, U., & Peerschke, E. I. (2014). Soluble gC1qR is an autocrine signal that induces B1R expression on endothelial cells. *J Immunol*, *192*(1), 377-384. https://doi.org/10.4049/jimmunol.1302031

Ghebrehiwet, B., Lim, B. L., Peerschke, E. I., Willis, A. C., & Reid, K. B. (1994). Isolation, cDNA cloning, and overexpression of a 33-kD cell surface glycoprotein that binds to the globular "heads" of C1q. *J Exp Med*, *179*(6), 1809-1821. https://doi.org/10.1084/jem.179.6.1809

Ghebrehiwet, B., Zaniewski, M., Fernandez, A., DiGiovanni, M., Reyes, T. N., Ji, P., Savitt, A. G., Williams, J. L., Seeliger, M. A., & Peerschke, E. I. B. (2024). The C1q and gC1qR

axis as a novel checkpoint inhibitor in cancer. *Front Immunol*, *15*, 1351656. https://doi.org/10.3389/fimmu.2024.1351656

Ghumra, A., Semblat, J. P., Ataide, R., Kifude, C., Adams, Y., Claessens, A., Anong, D. N., Bull, P. C., Fennell, C., Arman, M., Amambua-Ngwa, A., Walther, M., Conway, D. J., Kassambara, L., Doumbo, O. K., Raza, A., & Rowe, J. A. (2012). Induction of strain-transcending antibodies against Group A PfEMP1 surface antigens from virulent malaria parasites. *PLoS Pathog*, *8*(4), e1002665. https://doi.org/10.1371/journal.ppat.1002665

Gillrie, M. R., Avril, M., Brazier, A. J., Davis, S. P., Stins, M. F., Smith, J. D., & Ho, M. (2015). Diverse functional outcomes of Plasmodium falciparum ligation of EPCR: potential implications for malarial pathogenesis. *Cell Microbiol*, *17*(12), 1883-1899. https://doi.org/10.1111/cmi.12479

Gillrie, M. R., Renaux, B., Russell-Goldman, E., Avril, M., Brazier, A. J., Mihara, K., Di Cera, E., Milner, D. A., Jr., Hollenberg, M. D., Smith, J. D., & Ho, M. (2016). Thrombin Cleavage of Plasmodium falciparum Erythrocyte Membrane Protein 1 Inhibits Cytoadherence. *mBio*, *7*(5). https://doi.org/10.1128/mBio.01120-16

Goncalves, B. P., Huang, C. Y., Morrison, R., Holte, S., Kabyemela, E., Prevots, D. R., Fried, M., & Duffy, P. E. (2014). Parasite burden and severity of malaria in Tanzanian children. *N Engl J Med*, *370*(19), 1799-1808. https://doi.org/10.1056/NEJMoa1303944

Gotoh, K., Morisaki, T., Setoyama, D., Sasaki, K., Yagi, M., Igami, K., Mizuguchi, S., Uchiumi, T., Fukui, Y., & Kang, D. (2018). Mitochondrial p32/C1qbp Is a Critical Regulator of Dendritic Cell Metabolism and Maturation. *Cell Rep*, *25*(7), 1800-1815 e1804. https://doi.org/10.1016/j.celrep.2018.10.057

Grau, G. E., Mackenzie, C. D., Carr, R. A., Redard, M., Pizzolato, G., Allasia, C., Cataldo, C., Taylor, T. E., & Molyneux, M. E. (2003). Platelet accumulation in brain microvessels in fatal pediatric cerebral malaria. *J Infect Dis*, *187*(3), 461-466. https://doi.org/10.1086/367960

Greve, B., Kremsner, P. G., Lell, B., Luckner, D., & Schmid, D. (2000). Malarial anaemia in African children associated with high oxygen-radical production. *Lancet*, *355*(9197), 40-41. https://doi.org/10.1016/s0140-6736(99)04761-3

Griffin, J. T., Hollingsworth, T. D., Reyburn, H., Drakeley, C. J., Riley, E. M., & Ghani, A. C. (2015). Gradual acquisition of immunity to severe malaria with increasing exposure. *Proc Biol Sci*, *282*(1801), 20142657. https://doi.org/10.1098/rspb.2014.2657

Griffiths, M. J., Ndungu, F., Baird, K. L., Muller, D. P., Marsh, K., & Newton, C. R. (2001). Oxidative stress and erythrocyte damage in Kenyan children with severe Plasmodium falciparum malaria. *Br J Haematol*, *113*(2), 486-491. https://doi.org/10.1046/j.1365-2141.2001.02758.x

Gullingsrud, J., Saveria, T., Amos, E., Duffy, P. E., & Oleinikov, A. V. (2013). Structure-function-immunogenicity studies of PfEMP1 domain DBL2betaPF11_0521, a malaria parasite ligand for ICAM-1. *PLoS One*, *8*(4), e61323. https://doi.org/10.1371/journal.pone.0061323

Guo, N., Weremowicz, S., Lynch, N., Lim, B. L., Schwaeble, W., Peerschke, E. I., Morton, C. C., Reid, K. B., Ghebrehiwet, B., & Sastry, K. N. (1997). Assignment of C1QBP encoding the C1q globular domain binding protein (gC1q-R) to human chromosome 17 band p13.3 by in situ hybridization. *Cytogenet Cell Genet*, *77*(3-4), 283-284. https://doi.org/10.1159/000134598

Gupta, S., Snow, R. W., Donnelly, C. A., Marsh, K., & Newbold, C. (1999). Immunity to non-cerebral severe malaria is acquired after one or two infections. *Nat Med*, *5*(3), 340-343. https://doi.org/10.1038/6560

Hanson, J., Lam, S. W., 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., & Dondorp, A. M. (2012). Relative contributions of macrovascular and microvascular dysfunction to disease severity in falciparum malaria. *J Infect Dis*, *206*(4), 571-579. https://doi.org/10.1093/infdis/jis400

Hanson, J., Lee, S. J., Hossain, M. A., Anstey, N. M., Charunwatthana, P., Maude, R. J., Kingston, H. W., Mishra, S. K., Mohanty, S., Plewes, K., Piera, K., Hassan, M. U., Ghose, A., Faiz, M. A., White, N. J., Day, N. P., & Dondorp, A. M. (2015). Microvascular obstruction and endothelial activation are independently associated with the clinical manifestations of severe falciparum malaria in adults: an observational study. *BMC Med*, *13*, 122. https://doi.org/10.1186/s12916-015-0365-9

Hatabu, T., Kawazu, S., Aikawa, M., & Kano, S. (2003). Binding of Plasmodium falciparum-infected erythrocytes to the membrane-bound form of Fractalkine/CX3CL1. *Proc Natl Acad Sci U S A*, *100*(26), 15942-15946. https://doi.org/10.1073/pnas.2534560100

Heddini, A., Pettersson, F., Kai, O., Shafi, J., Obiero, J., Chen, Q., Barragan, A., Wahlgren, M., & Marsh, K. (2001). Fresh isolates from children with severe Plasmodium falciparum malaria bind to multiple receptors. *Infect Immun*, *69*(9), 5849-5856. https://doi.org/10.1128/IAI.69.9.5849-5856.2001

Helmbrecht, H., Lin, T. J., Janakiraman, S., Decker, K., & Nance, E. (2023). Prevalence and practices of immunofluorescent cell image processing: a systematic review. *Front Cell Neurosci*, *17*, 1188858. https://doi.org/10.3389/fncel.2023.1188858

Hendriksen, I. C., Mwanga-Amumpaire, J., von Seidlein, L., Mtove, G., White, L. J., Olaosebikan, R., Lee, S. J., Tshefu, A. K., Woodrow, C., Amos, B., Karema, C., Saiwaew, S., Maitland, K., Gomes, E., Pan-Ngum, W., Gesase, S., Silamut, K., Reyburn, H., Joseph, S., . . . Dondorp, A. M. (2012). Diagnosing severe falciparum malaria in parasitaemic African children: a prospective evaluation of plasma PfHRP2 measurement. *PLoS Med*, *9*(8), e1001297. https://doi.org/10.1371/journal.pmed.1001297

Hendriksen, I. C., White, L. J., Veenemans, J., Mtove, G., Woodrow, C., Amos, B., Saiwaew, S., Gesase, S., Nadjm, B., Silamut, K., Joseph, S., Chotivanich, K., Day, N. P., von Seidlein, L., Verhoef, H., Reyburn, H., White, N. J., & Dondorp, A. M. (2013). Defining falciparum-malaria-attributable severe febrile illness in moderate-to-high transmission settings on the basis of plasma PfHRP2 concentration. *J Infect Dis*, *207*(2), 351-361. https://doi.org/10.1093/infdis/jis675

Herrera, R., Anderson, C., Kumar, K., Molina-Cruz, A., Nguyen, V., Burkhardt, M., Reiter, K., Shimp, R., Jr., Howard, R. F., Srinivasan, P., Nold, M. J., Ragheb, D., Shi, L., DeCotiis, M., Aebig, J., Lambert, L., Rausch, K. M., Muratova, O., Jin, A., . . . Narum, D. L. (2015). Reversible Conformational Change in the Plasmodium falciparum Circumsporozoite Protein Masks Its Adhesion Domains. *Infect Immun*, *83*(10), 3771-3780. https://doi.org/10.1128/IAI.02676-14

Herwald, H., Dedio, J., Kellner, R., Loos, M., & Müller-Esterl, W. (1996). Isolation and characterization of the kininogen-binding protein p33 from endothelial cells. Identity with the gC1q receptor. *J Biol Chem*, *271*(22), 13040-13047. https://doi.org/10.1074/jbc.271.22.13040

Hsieh, F. L., Turner, L., Bolla, J. R., Robinson, C. V., Lavstsen, T., & Higgins, M. K. (2016). The structural basis for CD36 binding by the malaria parasite. *Nat Commun*, *7*, 12837. https://doi.org/10.1038/ncomms12837

Hughes, K. R., Biagini, G. A., & Craig, A. G. (2010). Continued cytoadherence of Plasmodium falciparum infected red blood cells after antimalarial treatment. *Mol Biochem Parasitol*, *169*(2), 71-78. https://doi.org/10.1016/j.molbiopara.2009.09.007

Jakeman, G. N., Saul, A., Hogarth, W. L., & Collins, W. E. (1999). Anaemia of acute malaria infections in non-immune patients primarily results from destruction of uninfected erythrocytes. *Parasitology*, *119 (Pt 2)*, 127-133. https://doi.org/10.1017/s0031182099004564

Jambou, R., Combes, V., Jambou, M. J., Weksler, B. B., Couraud, P. O., & Grau, G. E. (2010). Plasmodium falciparum adhesion on human brain microvascular endothelial cells involves transmigration-like cup formation and induces opening of intercellular junctions. *PLoS Pathog*, *6*(7), e1001021. https://doi.org/10.1371/journal.ppat.1001021

Janes, J. H., Wang, C. P., Levin-Edens, E., Vigan-Womas, I., Guillotte, M., Melcher, M., Mercereau-Puijalon, O., & Smith, J. D. (2011). Investigating the host binding signature on the Plasmodium falciparum PfEMP1 protein family. *PLoS Pathog*, *7*(5), e1002032. https://doi.org/10.1371/journal.ppat.1002032

Jensen, A. T., 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., & Theander, T. G. (2004). Plasmodium falciparum associated with severe childhood malaria preferentially expresses PfEMP1 encoded by group A var genes. *J Exp Med*, *199*(9), 1179-1190. https://doi.org/10.1084/jem.20040274

Jiang, J., Zhang, Y., Krainer, A. R., & Xu, R. M. (1999). Crystal structure of human p32, a doughnut-shaped acidic mitochondrial matrix protein. *Proc Natl Acad Sci U S A*, *96*(7), 3572-3577. https://doi.org/10.1073/pnas.96.7.3572

Joseph, K., Ghebrehiwet, B., Peerschke, E. I., Reid, K. B., & Kaplan, A. P. (1996). Identification of the zinc-dependent endothelial cell binding protein for high molecular weight kininogen and factor XII: identity with the receptor that binds to the globular "heads" of C1q (gC1q-R). *Proc Natl Acad Sci U S A*, *93*(16), 8552-8557. https://doi.org/10.1073/pnas.93.16.8552

Juillerat, A., Lewit-Bentley, A., Guillotte, M., Gangnard, S., Hessel, A., Baron, B., Vigan-Womas, I., England, P., Mercereau-Puijalon, O., & Bentley, G. A. (2011). Structure of a Plasmodium falciparum PfEMP1 rosetting domain reveals a role for the N-terminal segment in heparin-mediated rosette inhibition. *Proc Natl Acad Sci U S A*, *108*(13), 5243-5248. https://doi.org/10.1073/pnas.1018692108

Kariuki, S. M., Gitau, E., Gwer, S., Karanja, H. K., Chengo, E., Kazungu, M., Urban, B. C., & Newton, C. R. (2014). Value of Plasmodium falciparum histidine-rich protein 2 level and malaria retinopathy in distinguishing cerebral malaria from other acute encephalopathies in Kenyan children. *J Infect Dis*, *209*(4), 600-609. https://doi.org/10.1093/infdis/jit500

Karunaweera, N. D., Grau, G. E., Gamage, P., Carter, R., & Mendis, K. N. (1992). Dynamics of fever and serum levels of tumor necrosis factor are closely associated during clinical paroxysms in Plasmodium vivax malaria. *Proc Natl Acad Sci U S A*, *89*(8), 3200-3203. https://doi.org/10.1073/pnas.89.8.3200

Kraemer, S. M., & Smith, J. D. (2003). Evidence for the importance of genetic structuring to the structural and functional specialization of the Plasmodium falciparum var gene family. *Mol Microbiol*, *50*(5), 1527-1538. https://doi.org/10.1046/j.1365-2958.2003.03814.x

Kremsner, P. G., Adegnika, A. A., Hounkpatin, A. B., Zinsou, J. F., Taylor, T. E., Chimalizeni, Y., Liomba, A., Kombila, M., Bouyou-Akotet, M. K., Mawili Mboumba, D. P., Agbenyega, T., Ansong, D., Sylverken, J., Ogutu, B. R., Otieno, G. A., Wangwe, A., Bojang, K. A., Okomo, U., Sanya-Isijola, F., . . . Krishna, S. (2016). Intramuscular Artesunate for Severe Malaria in African Children: A Multicenter Randomized

Controlled Trial. *PLoS Med*, *13*(1), e1001938. https://doi.org/10.1371/journal.pmed.1001938

Kwiatkowski, D. (1990). Tumour necrosis factor, fever and fatality in falciparum malaria. *Immunol Lett*, *25*(1-3), 213-216. https://doi.org/10.1016/0165-2478(90)90117-9

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., & Rowe, J. A. (2006). Differential var gene transcription in Plasmodium falciparum isolates from patients with cerebral malaria compared to hyperparasitaemia. *Mol Biochem Parasitol*, *150*(2), 211-218. https://doi.org/10.1016/j.molbiopara.2006.08.005

Lambros, C., & Vanderberg, J. P. (1979). Synchronization of Plasmodium falciparum erythrocytic stages in culture. *J Parasitol*, *65*(3), 418-420.

Lamikanra, A. A., Merryweather-Clarke, A. T., Tipping, A. J., & Roberts, D. J. (2015). Distinct mechanisms of inadequate erythropoiesis induced by tumor necrosis factor alpha or malarial pigment. *PLoS One*, *10*(3), e0119836. https://doi.org/10.1371/journal.pone.0119836

Lamikanra, A. A., Theron, M., Kooij, T. W., & Roberts, D. J. (2009). Hemozoin (malarial pigment) directly promotes apoptosis of erythroid precursors. *PLoS One*, *4*(12), e8446. https://doi.org/10.1371/journal.pone.0008446

Lau, C. K., Turner, L., Jespersen, J. S., Lowe, E. D., Petersen, B., Wang, C. W., Petersen, J. E., Lusingu, J., Theander, T. G., Lavstsen, T., & Higgins, M. K. (2015). Structural conservation despite huge sequence diversity allows EPCR binding by the PfEMP1 family implicated in severe childhood malaria. *Cell Host Microbe*, *17*(1), 118-129. https://doi.org/10.1016/j.chom.2014.11.007

Lavstsen, T., Salanti, A., Jensen, A. T., Arnot, D. E., & Theander, T. G. (2003). Sub-grouping of Plasmodium falciparum 3D7 var genes based on sequence analysis of coding and non-coding regions. *Malar J*, *2*, 27. https://doi.org/10.1186/1475-2875-2-27

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., Lusingu, J., & Theander, T. G. (2012). Plasmodium falciparum erythrocyte membrane protein 1 domain cassettes 8 and 13 are associated with severe malaria in children. *Proc Natl Acad Sci U S A*, *109*(26), E1791-1800. https://doi.org/10.1073/pnas.1120455109

Lennartz, F., Adams, Y., Bengtsson, A., Olsen, R. W., Turner, L., Ndam, N. T., Ecklu-Mensah, G., Moussiliou, A., Ofori, M. F., Gamain, B., Lusingu, J. P., Petersen, J. E., Wang, C. W., Nunes-Silva, S., Jespersen, J. S., Lau, C. K., Theander, T. G., Lavstsen, T., Hviid, L., . . . Jensen, A. T. (2017). Structure-Guided Identification of a Family of Dual Receptor-Binding PfEMP1 that Is Associated with Cerebral Malaria. *Cell Host Microbe*, *21*(3), 403-414. https://doi.org/10.1016/j.chom.2017.02.009

Lennartz, F., Smith, C., Craig, A. G., & Higgins, M. K. (2019). Structural insights into diverse modes of ICAM-1 binding by Plasmodium falciparum-infected erythrocytes. *Proc Natl Acad Sci U S A*, *116*(40), 20124-20134. https://doi.org/10.1073/pnas.1911900116

Lewallen, S., White, V. A., Whitten, R. O., Gardiner, J., Hoar, B., Lindley, J., Lochhead, J., McCormick, A., Wade, K., Tembo, M., Mwenechanyana, J., Molyneux, M. E., & Taylor, T. E. (2000). Clinical-histopathological correlation of the abnormal retinal vessels in cerebral malaria. *Arch Ophthalmol*, *118*(7), 924-928.

Lim, B. L., Reid, K. B., Ghebrehiwet, B., Peerschke, E. I., Leigh, L. A., & Preissner, K. T. (1996). The binding protein for globular heads of complement C1q, gC1qR. Functional expression and characterization as a novel vitronectin binding factor. *J Biol Chem*, *271*(43), 26739-26744. https://doi.org/10.1074/jbc.271.43.26739

Lin, Y., Tebulo, A., Small, D., Seydel, K., Taylor, T., & Zhang, B. (2023). Using Malarial Retinopathy to Improve the Diagnosis of Pediatric Cerebral Malaria. *Am J Trop Med Hyg*, *108*(1), 69-75. https://doi.org/10.4269/ajtmh.22-0547

Liu, M., Solomon, W., Cespedes, J. C., Wilson, N. O., Ford, B., & Stiles, J. K. (2018). Neuregulin-1 attenuates experimental cerebral malaria (ECM) pathogenesis by regulating ErbB4/AKT/STAT3 signaling. *J Neuroinflammation*, *15*(1), 104. https://doi.org/10.1186/s12974-018-1147-z

Looareesuwan, S., Davis, T. M., Pukrittayakamee, S., Supanaranond, W., Desakorn, V., Silamut, K., Krishna, S., Boonamrung, S., & White, N. J. (1991). Erythrocyte survival in severe falciparum malaria. *Acta Trop*, *48*(4), 263-270. https://doi.org/10.1016/0001-706x(91)90014-b

Lu, P. D., Galanakis, D. K., Ghebrehiwet, B., & Peerschke, E. I. (1999). The receptor for the globular "heads" of C1q, gC1q-R, binds to fibrinogen/fibrin and impairs its polymerization. *Clin Immunol*, *90*(3), 360-367. https://doi.org/10.1006/clim.1998.4660

Luo, Y., Yu, H., & Peterlin, B. M. (1994). Cellular protein modulates effects of human immunodeficiency virus type 1 Rev. *J Virol*, *68*(6), 3850-3856. https://doi.org/10.1128/jvi.68.6.3850-3856.1994

MacCormick, I. J., Beare, N. A., Taylor, T. E., Barrera, V., White, V. A., Hiscott, P., Molyneux, M. E., Dhillon, B., & Harding, S. P. (2014). Cerebral malaria in children: using the retina to study the brain. *Brain*, *137*(Pt 8), 2119-2142. https://doi.org/10.1093/brain/awu001

MacPherson, G. G., Warrell, M. J., White, N. J., Looareesuwan, S., & Warrell, D. A. (1985). Human cerebral malaria. A quantitative ultrastructural analysis of parasitized erythrocyte sequestration. *Am J Pathol*, *119*(3), 385-401.

Madkhali, A. M., Alkurbi, M. O., Szestak, T., Bengtsson, A., Patil, P. R., Wu, Y., Al-Harthi, S. A., Jensen, A. T., Pleass, R., & 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), e111518. https://doi.org/10.1371/journal.pone.0111518

Magallon-Tejada, A., Machevo, S., Cistero, P., Lavstsen, T., Aide, P., Rubio, M., Jimenez, A., Turner, L., Valmaseda, A., Gupta, H., De Las Salas, B., Mandomando, I., Wang, C. W., Petersen, J. E., Munoz, J., Gascon, J., Macete, E., Alonso, P. L., Chitnis, C. E., . . . Mayor, A. (2016). Cytoadhesion to gC1qR through Plasmodium falciparum Erythrocyte Membrane Protein 1 in Severe Malaria. *PLoS Pathog*, *12*(11), e1006011. https://doi.org/10.1371/journal.ppat.1006011

Mahamar, A., Attaher, O., Swihart, B., Barry, A., Diarra, B. S., Kanoute, M. B., Cisse, K. B., Dembele, A. B., Keita, S., Gamain, B., Gaoussou, S., Issiaka, D., Dicko, A., Duffy, P. E., & Fried, M. (2017). Host factors that modify Plasmodium falciparum adhesion to endothelial receptors. *Sci Rep*, *7*(1), 13872. https://doi.org/10.1038/s41598-017-14351-7

Malhotra, R., & Sim, R. B. (1989). Chemical and hydrodynamic characterization of the human leucocyte receptor for complement subcomponent C1q. *Biochem J*, *262*(2), 625-631. https://doi.org/10.1042/bj2620625

Malleret, B., Claser, C., Ong, A. S., Suwanarusk, R., Sriprawat, K., Howland, S. W., Russell, B., Nosten, F., & Renia, L. (2011). A rapid and robust tri-color flow cytometry assay for monitoring malaria parasite development. *Sci Rep*, *1*, 118. https://doi.org/10.1038/srep00118

Manning, L., Laman, M., Stanisic, D., Rosanas-Urgell, A., Bona, C., Teine, D., Siba, P., Mueller, I., & Davis, T. M. (2011). Plasma Plasmodium falciparum histidine-rich

protein-2 concentrations do not reflect severity of malaria in Papua new guinean children. *Clin Infect Dis*, *52*(4), 440-446. https://doi.org/10.1093/cid/ciq105

Marlin, S. D., Staunton, D. E., Springer, T. A., Stratowa, C., Sommergruber, W., & Merluzzi, V. J. (1990). A soluble form of intercellular adhesion molecule-1 inhibits rhinovirus infection. *Nature*, *344*(6261), 70-72. https://doi.org/10.1038/344070a0

Marsh, K., Forster, D., Waruiru, C., Mwangi, I., Winstanley, M., Marsh, V., Newton, C., Winstanley, P., Warn, P., Peshu, N., & et al. (1995). Indicators of life-threatening malaria in African children. *N Engl J Med*, *332*(21), 1399-1404. https://doi.org/10.1056/nejm199505253322102

Marsh, K., & Snow, R. W. (1997). Host-parasite interaction and morbidity in malaria endemic areas. *Philos Trans R Soc Lond B Biol Sci*, *352*(1359), 1385-1394. https://doi.org/10.1098/rstb.1997.0124

Mayer, C., Slater, L., Erat, M. C., Konrat, R., & Vakonakis, I. (2012). Structural analysis of the Plasmodium falciparum erythrocyte membrane protein 1 (PfEMP1) intracellular domain reveals a conserved interaction epitope. *J Biol Chem*, *287*(10), 7182-7189. https://doi.org/10.1074/jbc.M111.330779

Mayor, A., Hafiz, A., Bassat, Q., Rovira-Vallbona, E., Sanz, S., Machevo, S., Aguilar, R., Cistero, P., Sigauque, B., Menendez, C., Alonso, P. L., & Chitnis, C. E. (2011). Association of severe malaria outcomes with platelet-mediated clumping and adhesion to a novel host receptor. *PLoS One*, *6*(4), e19422. https://doi.org/10.1371/journal.pone.0019422

McLean, F. E., Azasi, Y., Sutherland, C., Toboh, E., Ansong, D., Agbenyega, T., Awandare, G., & Rowe, J. A. (2024). Detection of naturally acquired, strain-transcending antibodies against rosetting Plasmodium falciparum strains in humans. *Infect Immun*, *92*(7), e0001524. https://doi.org/10.1128/iai.00015-24

McLean, F. E., Omondi, B. R., Diallo, N., Otoboh, S., Kifude, C., Abdi, A. I., Lim, R., Otto, T. D., Ghumra, A., & Rowe, J. A. (2025). Identification of novel PfEMP1 variants containing domain cassettes 11, 15 and 8 that mediate the Plasmodium falciparum virulence-associated rosetting phenotype. *PLoS Pathog*, *21*(1), e1012434. https://doi.org/10.1371/journal.ppat.1012434

Miller, L. H., Baruch, D. I., Marsh, K., & Doumbo, O. K. (2002). The pathogenic basis of malaria. *Nature*, *415*(6872), 673-679. https://doi.org/10.1038/415673a

Miller, L. H., Good, M. F., & Milon, G. (1994). Malaria pathogenesis. *Science*, *264*(5167), 1878-1883. https://doi.org/10.1126/science.8009217

Milner, D. A., Jr., Lee, J. J., Frantzreb, C., Whitten, R. O., Kamiza, S., Carr, R. A., Pradham, A., Factor, R. E., Playforth, K., Liomba, G., Dzamalala, C., Seydel, K. B., Molyneux, M. E., & Taylor, T. E. (2015). Quantitative Assessment of Multiorgan Sequestration of Parasites in Fatal Pediatric Cerebral Malaria. *J Infect Dis*, *212*(8), 1317-1321. https://doi.org/10.1093/infdis/jiv205

Milner, D. A., Jr., Whitten, R. O., Kamiza, S., Carr, R., Liomba, G., Dzamalala, C., Seydel, K. B., Molyneux, M. E., & Taylor, T. E. (2014). The systemic pathology of cerebral malaria in African children. *Front Cell Infect Microbiol*, *4*, 104. https://doi.org/10.3389/fcimb.2014.00104

Molyneux, M. E. (1990). Cerebral malaria in children: clinical implications of cytoadherence. *Am J Trop Med Hyg*, *43*(2 Pt 2), 38-41. https://doi.org/10.4269/ajtmh.1990.43.38

Mosnier, L. O., Zlokovic, B. V., & Griffin, J. H. (2007). The cytoprotective protein C pathway. *Blood*, *109*(8), 3161-3172. https://doi.org/10.1182/blood-2006-09-003004

Moxon, C. A., Wassmer, S. C., Milner, D. A., Jr., 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., & 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), 842-851. https://doi.org/10.1182/blood-2013-03-490219

Mu, J., Awadalla, P., Duan, J., McGee, K. M., Joy, D. A., McVean, G. A., & Su, X. Z. (2005). Recombination hotspots and population structure in Plasmodium falciparum. *PLoS Biol*, *3*(10), e335. https://doi.org/10.1371/journal.pbio.0030335

Muta, T., Kang, D., Kitajima, S., Fujiwara, T., & Hamasaki, N. (1997). p32 protein, a splicing factor 2-associated protein, is localized in mitochondrial matrix and is functionally important in maintaining oxidative phosphorylation. *J Biol Chem*, *272*(39), 24363-24370. https://doi.org/10.1074/jbc.272.39.24363

Navone, S. E., Marfia, G., Invernici, G., Cristini, S., Nava, S., Balbi, S., Sangiorgi, S., Ciusani, E., Bosutti, A., Alessandri, G., Slevin, M., & Parati, E. A. (2013). Isolation and expansion of human and mouse brain microvascular endothelial cells. *Nat Protoc*, *8*(9), 1680-1693. https://doi.org/10.1038/nprot.2013.107

Navratil, E., Couvelard, A., Rey, A., Hénin, D., & Scoazec, J. Y. (2003). Expression of cell adhesion molecules by microvascular endothelial cells in the cortical and subcortical regions of the normal human brain: an immunohistochemical analysis. *Neuropathology and Applied Neurobiology*, *23*(1), 68-80. https://doi.org/10.1111/j.1365-2990.1997.tb01187.x

Ndonwi, M., Burlingame, O. O., Miller, A. S., Tollefsen, D. M., Broze, G. J., Jr., & Goldberg, D. E. (2011). Inhibition of antithrombin by Plasmodium falciparum histidine-rich protein II. *Blood*, *117*(23), 6347-6354. https://doi.org/10.1182/blood-2010-12-326876

Newbold, C., Warn, P., Black, G., Berendt, A., Craig, A., Snow, B., Msobo, M., Peshu, N., & Marsh, K. (1997). Receptor-specific adhesion and clinical disease in Plasmodium falciparum. *Am J Trop Med Hyg*, *57*(4), 389-398. https://doi.org/10.4269/ajtmh.1997.57.389

Newton, C. R., Taylor, T. E., & Whitten, R. O. (1998). Pathophysiology of fatal falciparum malaria in African children. *Am J Trop Med Hyg*, *58*(5), 673-683. https://doi.org/10.4269/ajtmh.1998.58.673

O'Brien, N. F., Fonseca, Y., Johnson, H. C., Postels, D., Birbeck, G. L., Chimalizeni, Y., Seydel, K. B., Bernard Gushu, M., Phiri, T., June, S., Chetcuti, K., Vidal, L., Goyal, M. S., & Taylor, T. E. (2022). Mechanisms of Transcranial Doppler Ultrasound phenotypes in paediatric cerebral malaria remain elusive. *Malar J*, *21*(1), 196. https://doi.org/10.1186/s12936-022-04163-0

Obeng-Adjei, N., Larremore, D. B., Turner, L., Ongoiba, A., Li, S., Doumbo, S., Yazew, T. B., Kayentao, K., Miller, L. H., Traore, B., Pierce, S. K., Buckee, C. O., Lavstsen, T., Crompton, P. D., & Tran, T. M. (2020). Longitudinal analysis of naturally acquired PfEMP1 CIDR domain variant antibodies identifies associations with malaria protection. *JCI Insight*, *5*(12). https://doi.org/10.1172/jci.insight.137262

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., & Craig, A. G. (2011). Specific receptor usage in Plasmodium falciparum cytoadherence is associated with disease outcome. *PLoS One*, *6*(3), e14741. https://doi.org/10.1371/journal.pone.0014741

Ockenhouse, C. F., Betageri, R., Springer, T. A., & Staunton, D. E. (1992). Plasmodium falciparum-infected erythrocytes bind ICAM-1 at a site distinct from LFA-1, Mac-1, and human rhinovirus. *Cell*, *68*(1), 63-69. https://doi.org/10.1016/0092-8674(92)90206-r

Ockenhouse, C. F., Tegoshi, T., Maeno, Y., Benjamin, C., Ho, M., Kan, K. E., Thway, Y., Win, K., Aikawa, M., & Lobb, R. R. (1992). Human vascular endothelial cell adhesion receptors for Plasmodium falciparum-infected erythrocytes: roles for endothelial

leukocyte adhesion molecule 1 and vascular cell adhesion molecule 1. *J Exp Med*, *176*(4), 1183-1189. https://doi.org/10.1084/jem.176.4.1183

Oleinikov, A. V., Amos, E., Frye, I. T., Rossnagle, E., Mutabingwa, T. K., Fried, M., & 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 Pathog*, *5*(4), e1000386. https://doi.org/10.1371/journal.ppat.1000386

Opi, D. H., Ndila, C. M., Uyoga, S., Macharia, A. W., Fennell, C., Ochola, L. B., Nyutu, G., Siddondo, B. R., Ojal, J., Shebe, M., Awuondo, K. O., Mturi, N., Peshu, N., Tsofa, B., Band, G., Maitland, K., Kwiatkowski, D. P., Rockett, K. A., Williams, T. N., & Rowe, J. A. (2023). Non-O ABO blood group genotypes differ in their associations with Plasmodium falciparum rosetting and severe malaria. *PLoS Genet*, *19*(9), e1010910. https://doi.org/10.1371/journal.pgen.1010910

Opi, D. H., Ochola, L. B., Tendwa, M., Siddondo, B. R., Ocholla, H., Fanjo, H., Ghumra, A., Ferguson, D. J., Rowe, J. A., & Williams, T. N. (2014). Mechanistic Studies of the Negative Epistatic Malaria-protective Interaction Between Sickle Cell Trait and alpha(+)thalassemia. *EBioMedicine*, *1*(1), 29-36. https://doi.org/10.1016/j.ebiom.2014.10.006

Oquendo, P., Hundt, E., Lawler, J., & Seed, B. (1989). CD36 directly mediates cytoadherence of Plasmodium falciparum parasitized erythrocytes. *Cell*, *58*(1), 95-101. https://doi.org/10.1016/0092-8674(89)90406-6

Ortolan, L. S., Avril, M., Xue, J., Seydel, K. B., Zheng, Y., & Smith, J. D. (2022). Plasmodium falciparum Parasite Lines Expressing DC8 and Group A PfEMP1 Bind to Brain, Intestinal, and Kidney Endothelial Cells. *Front Cell Infect Microbiol*, *12*, 813011. https://doi.org/10.3389/fcimb.2022.813011

Otto, T. D., Assefa, S. A., Bohme, U., Sanders, M. J., Kwiatkowski, D., Pf3k, c., Berriman, M., & Newbold, C. (2019). Evolutionary analysis of the most polymorphic gene family in falciparum malaria. *Wellcome Open Res*, *4*, 193. https://doi.org/10.12688/wellcomeopenres.15590.1

Pain, A., Ferguson, D. J., Kai, O., Urban, B. C., Lowe, B., Marsh, K., & Roberts, D. J. (2001). Platelet-mediated clumping of Plasmodium falciparum-infected erythrocytes is a common adhesive phenotype and is associated with severe malaria. *Proc Natl Acad Sci U S A*, *98*(4), 1805-1810. https://doi.org/10.1073/pnas.98.4.1805

Park, G. S., Opoka, R. O., Shabani, E., Wypyszynski, A., Hanisch, B., & John, C. C. (2017). Plasmodium falciparum Histidine-Rich Protein-2 Plasma Concentrations Are Higher in Retinopathy-Negative Cerebral Malaria Than in Severe Malarial Anemia. *Open Forum Infect Dis*, *4*(3), ofx151. https://doi.org/10.1093/ofid/ofx151

Pasvol, G., Wilson, R. J., Smalley, M. E., & Brown, J. (1978). Separation of viable schizont-infected red cells of Plasmodium falciparum from human blood. *Ann Trop Med Parasitol*, *72*(1), 87-88. https://doi.org/10.1080/00034983.1978.11719283

Peerschke, E. I., & Ghebrehiwet, B. (1987). Human blood platelets possess specific binding sites for C1q. *J Immunol*, *138*(5), 1537-1541.

Peerschke, E. I., Smyth, S. S., Teng, E. I., Dalzell, M., & Ghebrehiwet, B. (1996). Human umbilical vein endothelial cells possess binding sites for the globular domain of C1q. *J Immunol*, *157*(9), 4154-4158.

Petersen, J. E. V., Saelens, J. W., Freedman, E., Turner, L., Lavstsen, T., Fairhurst, R. M., Diakite, M., & Taylor, S. M. (2021). Sickle-trait hemoglobin reduces adhesion to both CD36 and EPCR by Plasmodium falciparum-infected erythrocytes. *PLoS Pathog*, *17*(6), e1009659. https://doi.org/10.1371/journal.ppat.1009659

Phan, Q. T., Solis, N. V., Lin, J., Swidergall, M., Singh, S., Liu, H., Sheppard, D. C., Ibrahim, A. S., Mitchell, A. P., & Filler, S. G. (2022). Serum bridging molecules drive candidal invasion of human but not mouse endothelial cells. *PLoS Pathog*, *18*(7), e1010681. https://doi.org/10.1371/journal.ppat.1010681

Ponsford, M. J., Medana, I. M., Prapansilp, P., Hien, T. T., Lee, S. J., Dondorp, A. M., Esiri, M. M., Day, N. P., White, N. J., & Turner, G. D. (2012). Sequestration and microvascular congestion are associated with coma in human cerebral malaria. *J Infect Dis*, *205*(4), 663-671. https://doi.org/10.1093/infdis/jir812

Pouvelle, B., Matarazzo, V., Jurzynski, C., Nemeth, J., Ramharter, M., Rougon, G., & Gysin, J. (2007). Neural cell adhesion molecule, a new cytoadhesion receptor for Plasmodium falciparum-infected erythrocytes capable of aggregation. *Infect Immun*, *75*(7), 3516-3522. https://doi.org/10.1128/IAI.01852-06

Qi, D., Lin, H., Hu, B., & Wei, Y. (2023). A review on in vitro model of the blood-brain barrier (BBB) based on hCMEC/D3 cells. *J Control Release*, *358*, 78-97. https://doi.org/10.1016/j.jconrel.2023.04.020

Rask, T. S., Hansen, D. A., Theander, T. G., Gorm Pedersen, A., & Lavstsen, T. (2010). Plasmodium falciparum erythrocyte membrane protein 1 diversity in seven genomes--divide and conquer. *PLoS Comput Biol*, *6*(9). https://doi.org/10.1371/journal.pcbi.1000933

Reyes, R. A., Raghavan, S. S. R., Hurlburt, N. K., Introini, V., Bol, S., Kana, I. H., Jensen, R. W., Martinez-Scholze, E., Gestal-Mato, M., Lopez-Gutierrez, B., Sanz, S., Bancells, C., Fernandez-Quintero, M. L., Loeffler, J. R., Ferguson, J. A., Lee, W. H., Martin, G. M., Theander, T. G., Lusingu, J. P. A., . . . Lavstsen, T. (2024). Broadly inhibitory antibodies to severe malaria virulence proteins. *Nature*, *636*(8041), 182-189. https://doi.org/10.1038/s41586-024-08220-3

Riganti, M., Pongponratn, E., Tegoshi, T., Looareesuwan, S., Punpoowong, B., & Aikawa, M. (1990). Human cerebral malaria in Thailand: a clinico-pathological correlation. *Immunol Lett*, *25*(1-3), 199-205. https://doi.org/10.1016/0165-2478(90)90115-7

Riley, E. M., Wagner, G. E., Akanmori, B. D., & Koram, K. A. (2001). Do maternally acquired antibodies protect infants from malaria infection? *Parasite Immunol*, *23*(2), 51-59. https://doi.org/10.1046/j.1365-3024.2001.00364.x

Roberts, D. D., Sherwood, J. A., Spitalnik, S. L., Panton, L. J., Howard, R. J., Dixit, V. M., Frazier, W. A., Miller, L. H., & Ginsburg, V. (1985). Thrombospondin binds falciparum malaria parasitized erythrocytes and may mediate cytoadherence. *Nature*, *318*(6041), 64-66. https://doi.org/10.1038/318064a0

Roberts, D. J., Craig, A. G., Berendt, A. R., Pinches, R., Nash, G., Marsh, K., & Newbold, C. I. (1992). Rapid switching to multiple antigenic and adhesive phenotypes in malaria. *Nature*, *357*(6380), 689-692. https://doi.org/10.1038/357689a0

Robson, K. J. H., Walliker, D., Creasey, A. M., & Wilson, R. J. M. (1993). Fcr-3, Chloroquine Resistance and Cross-Contamination of Culture Lines of Plasmodium-Falciparum - Reply. *Parasitology Today*, *9*(4), 127-127. https://doi.org/Doi 10.1016/0169-4758(93)90171-B

Rogerson, S. J., Chaiyaroj, S. C., Ng, K., Reeder, J. C., & Brown, G. V. (1995). Chondroitin sulfate A is a cell surface receptor for Plasmodium falciparum-infected erythrocytes. *J Exp Med*, *182*(1), 15-20. https://doi.org/10.1084/jem.182.1.15

Rogerson, S. J., & Milner Jr, D. A. (2009). Parasitic infections of the brain. In *Inflammatory Diseases of the Central Nervous System* (pp. 173-184). https://doi.org/10.1017/cbo9781139195805.014

Rowe, A., Obeiro, J., Newbold, C. I., & Marsh, K. (1995). Plasmodium falciparum rosetting is associated with malaria severity in Kenya. *Infect Immun*, *63*(6), 2323-2326. https://doi.org/10.1128/iai.63.6.2323-2326.1995

Rowe, J. A., Claessens, A., Corrigan, R. A., & Arman, M. (2009). Adhesion of Plasmodium falciparum-infected erythrocytes to human cells: molecular mechanisms and therapeutic implications. *Expert Rev Mol Med*, *11*, e16. https://doi.org/10.1017/S1462399409001082

Rowe, J. A., Handel, I. G., Thera, M. A., Deans, A. M., Lyke, K. E., Koné, A., Diallo, D. A., Raza, A., Kai, O., Marsh, K., Plowe, C. V., Doumbo, O. K., & Moulds, J. M. (2007). Blood group O protects against severe Plasmodium falciparum malaria through the mechanism of reduced rosetting. *Proc Natl Acad Sci U S A*, *104*(44), 17471-17476. https://doi.org/10.1073/pnas.0705390104

Rowe, J. A., Moulds, J. M., Newbold, C. I., & Miller, L. H. (1997). P. falciparum rosetting mediated by a parasite-variant erythrocyte membrane protein and complement-receptor 1. *Nature*, *388*(6639), 292-295. https://doi.org/10.1038/40888

Rubach, M. P., Mukemba, J., Florence, S., John, B., Crookston, B., Lopansri, B. K., Yeo, T. W., Piera, K. A., Alder, S. C., Weinberg, J. B., Anstey, N. M., Granger, D. L., & Mwaikambo, E. D. (2012). Plasma Plasmodium falciparum histidine-rich protein-2 concentrations are associated with malaria severity and mortality in Tanzanian children. *PLoS One*, *7*(5), e35985. https://doi.org/10.1371/journal.pone.0035985

Sahu, P. K., Duffy, F. J., Dankwa, S., Vishnyakova, M., Majhi, M., Pirpamer, L., Vigdorovich, V., Bage, J., Maharana, S., Mandala, W., Rogerson, S. J., Seydel, K. B., Taylor, T. E., Kim, K., Sather, D. N., Mohanty, A., Mohanty, R. R., Mohanty, A., Pattnaik, R., . . . Wassmer, S. C. (2021). Determinants of brain swelling in pediatric and adult cerebral malaria. *JCI Insight*, *6*(18). https://doi.org/10.1172/jci.insight.145823

Sampath, S., Brazier, A. J., Avril, M., Bernabeu, M., Vigdorovich, V., Mascarenhas, A., Gomes, E., Sather, D. N., Esmon, C. T., & Smith, J. D. (2015). Plasmodium falciparum adhesion domains linked to severe malaria differ in blockade of endothelial protein C receptor. *Cell Microbiol*, *17*(12), 1868-1882. https://doi.org/10.1111/cmi.12478

Sanchez, C. P., Karathanasis, C., Sanchez, R., Cyrklaff, M., Jager, J., Buchholz, B., Schwarz, U. S., Heilemann, M., & Lanzer, M. (2019). Single-molecule imaging and quantification of the immune-variant adhesin VAR2CSA on knobs of Plasmodium falciparum-infected erythrocytes. *Commun Biol*, *2*, 172. https://doi.org/10.1038/s42003-019-0429-z

Schmidt, J. A., Udeinya, I. J., Leech, J. H., Hay, R. J., Aikawa, M., Barnwell, J., Green, I., & Miller, L. H. (1982). Plasmodium falciparum malaria. An amelanotic melanoma cell line bears receptors for the knob ligand on infected erythrocytes. *J Clin Invest*, *70*(2), 379-386. https://doi.org/10.1172/jci110627

Seydel, K. B., Kampondeni, S. D., Valim, C., Potchen, M. J., Milner, D. A., Muwalo, F. W., Birbeck, G. L., Bradley, W. G., Fox, L. L., Glover, S. J., Hammond, C. A., Heyderman, R. S., Chilingulo, C. A., Molyneux, M. E., & Taylor, T. E. (2015). Brain swelling and death in children with cerebral malaria. *N Engl J Med*, *372*(12), 1126-1137. https://doi.org/10.1056/NEJMoa1400116

Seydel, K. B., Milner, D. A., Jr., Kamiza, S. B., Molyneux, M. E., & Taylor, T. E. (2006). The distribution and intensity of parasite sequestration in comatose Malawian children. *J Infect Dis*, *194*(2), 208-205. https://doi.org/10.1086/505078

Siano, J. P., Grady, K. K., Millet, P., & Wick, T. M. (1998). Short report: Plasmodium falciparum: cytoadherence to alpha(v)beta3 on human microvascular endothelial cells. *Am J Trop Med Hyg*, *59*(1), 77-79. https://doi.org/10.4269/ajtmh.1998.59.77

Simmonds, R. E., & Lane, D. A. (1999). Structural and Functional Implications of the Intron/Exon Organization of the Human Endothelial Cell Protein C/Activated Protein C Receptor (EPCR) Gene: Comparison With the Structure of CD1/Major Histocompatibility Complex □1 and □2 Domains. *Blood*, *94*(2), 632-641. https://doi.org/10.1182/blood.V94.2.632

Smith, H., Nelson, J. A., Gahmberg, C. G., Crandall, I., & Sherman, I. W. (1992). Plasmodium falciparum: cytoadherence of malaria-infected erythrocytes to human brain capillary and umbilical vein endothelial cells--a comparative study of adhesive ligands. *Exp Parasitol*, *75*(3), 269-280. https://doi.org/10.1016/0014-4894(92)90212-s

Smith, J. D., Chitnis, C. E., Craig, A. G., Roberts, D. J., Hudson-Taylor, D. E., Peterson, D. S., Pinches, R., Newbold, C. I., & 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), 101-110. https://doi.org/10.1016/0092-8674(95)90056-x

Smith, J. D., Subramanian, G., Gamain, B., Baruch, D. I., & Miller, L. H. (2000). Classification of adhesive domains in the Plasmodium falciparum erythrocyte membrane protein 1 family. *Mol Biochem Parasitol*, *110*(2), 293-310. https://doi.org/10.1016/s0166-6851(00)00279-6

Snow, R. W., Omumbo, J. A., Lowe, B., Molyneux, C. S., Obiero, J. O., Palmer, A., Weber, M. W., Pinder, M., Nahlen, B., Obonyo, C., Newbold, C., Gupta, S., & Marsh, K. (1997). Relation between severe malaria morbidity in children and level of Plasmodium falciparum transmission in Africa. *Lancet*, *349*(9066), 1650-1654. https://doi.org/10.1016/S0140-6736(97)02038-2

Staunton, D. E., Marlin, S. D., Stratowa, C., Dustin, M. L., & Springer, T. A. (1988). Primary structure of ICAM-1 demonstrates interaction between members of the immunoglobulin and integrin supergene families. *Cell*, *52*(6), 925-933. https://doi.org/10.1016/0092-8674(88)90434-5

Staunton, D. E., Merluzzi, V. J., Rothlein, R., Barton, R., Marlin, S. D., & Springer, T. A. (1989). A cell adhesion molecule, ICAM-1, is the major surface receptor for rhinoviruses. *Cell*, *56*(5), 849-853. https://doi.org/10.1016/0092-8674(89)90689-2

Storm, J., Jespersen, J. S., Seydel, K. B., Szestak, T., Mbewe, M., Chisala, N. V., Phula, P., Wang, C. W., Taylor, T. E., Moxon, C. A., Lavstsen, T., & Craig, A. G. (2019). Cerebral malaria is associated with differential cytoadherence to brain endothelial cells. *EMBO Mol Med*, *11*(2). https://doi.org/10.15252/emmm.201809164

Storm, J., Wu, Y., Davies, J., Moxon, C. A., & Craig, A. G. (2020). Testing the effect of PAR1 inhibitors on Plasmodium falciparum-induced loss of endothelial cell barrier function. *Wellcome Open Res*, *5*, 34. https://doi.org/10.12688/wellcomeopenres.15602.3

Sturm, A., Amino, R., van de Sand, C., Regen, T., Retzlaff, S., Rennenberg, A., Krueger, A., Pollok, J. M., Menard, R., & Heussler, V. T. (2006). Manipulation of host hepatocytes by the malaria parasite for delivery into liver sinusoids. *Science*, *313*(5791), 1287-1290. https://doi.org/10.1126/science.1129720

Su, X. Z., Heatwole, V. M., Wertheimer, S. P., Guinet, F., Herrfeldt, J. A., Peterson, D. S., Ravetch, J. A., & Wellems, T. E. (1995). The large diverse gene family var encodes proteins involved in cytoadherence and antigenic variation of Plasmodium falciparum-infected erythrocytes. *Cell*, *82*(1), 89-100. https://doi.org/10.1016/0092-8674(95)90055-1

Taylor, T. E., Fu, W. J., Carr, R. A., Whitten, R. O., Mueller, J. S., Fosiko, N. G., Lewallen, S., Liomba, N. G., & Molyneux, M. E. (2004). Differentiating the pathologies of cerebral malaria by postmortem parasite counts. *Nat Med*, *10*(2), 143-145. https://doi.org/10.1038/nm986

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., & Montgomery, J. (2014). Differential PfEMP1 expression is associated with cerebral malaria pathology. *PLoS Pathog*, *10*(12), e1004537. https://doi.org/10.1371/journal.ppat.1004537

Tenner, A. J., & Cooper, N. R. (1981). Identification of types of cells in human peripheral blood that bind C1q. *J Immunol*, *126*(3), 1174-1179.

Tessema, S. K., Nakajima, R., Jasinskas, A., Monk, S. L., Lekieffre, L., Lin, E., Kiniboro, B., Proietti, C., Siba, P., Felgner, P. L., Doolan, D. L., Mueller, I., & Barry, A. E. (2019). Protective Immunity against Severe Malaria in Children Is Associated with a Limited Repertoire of Antibodies to Conserved PfEMP1 Variants. *Cell Host Microbe*, *26*(5), 579-590 e575. https://doi.org/10.1016/j.chom.2019.10.012

Tonkin-Hill, G. Q., Trianty, L., Noviyanti, R., Nguyen, H. H. T., Sebayang, B. F., Lampah, D. A., Marfurt, J., Cobbold, S. A., Rambhatla, J. S., McConville, M. J., Rogerson, S. J., Brown, G. V., Day, K. P., Price, R. N., Anstey, N. M., Papenfuss, A. T., & Duffy, M. F. (2018). The Plasmodium falciparum transcriptome in severe malaria reveals altered expression of genes involved in important processes including surface antigen-encoding var genes. *PLoS Biol*, *16*(3), e2004328. https://doi.org/10.1371/journal.pbio.2004328

Trager, W. (1993). FCR-3, chloroquine resistance and cross-contamination of culture lines of Plasmodium falciparum. *Parasitol Today*, *9*(4), 127; author reply 127. https://doi.org/10.1016/0169-4758(93)90170-k

Treutiger, C. J., Heddini, A., Fernandez, V., Muller, W. A., & Wahlgren, M. (1997). PECAM-1/CD31, an endothelial receptor for binding Plasmodium falciparum-infected erythrocytes. *Nat Med*, *3*(12), 1405-1408. https://doi.org/10.1038/nm1297-1405

Treutiger, C. J., Hedlund, I., Helmby, H., Carlson, J., Jepson, A., Twumasi, P., Kwiatkowski, D., Greenwood, B. M., & Wahlgren, M. (1992). Rosette formation in Plasmodium falciparum isolates and anti-rosette activity of sera from Gambians with cerebral or uncomplicated malaria. *Am J Trop Med Hyg*, *46*(5), 503-510. https://doi.org/10.4269/ajtmh.1992.46.503

Tuikue Ndam, N., Moussiliou, A., Lavstsen, T., Kamaliddin, C., Jensen, A. T. R., Mama, A., Tahar, R., Wang, C. W., Jespersen, J. S., Alao, J. M., Gamain, B., Theander, T. G., & Deloron, P. (2017). Parasites Causing Cerebral Falciparum Malaria Bind Multiple Endothelial Receptors and Express EPCR and ICAM-1-Binding PfEMP1. *J Infect Dis*, *215*(12), 1918-1925. https://doi.org/10.1093/infdis/jix230

Turner, L., Lavstsen, T., Berger, S. S., Wang, C. W., Petersen, J. E., Avril, M., Brazier, A. J., Freeth, J., Jespersen, J. S., Nielsen, M. A., Magistrado, P., Lusingu, J., Smith, J. D., Higgins, M. K., & Theander, T. G. (2013). Severe malaria is associated with parasite binding to endothelial protein C receptor. *Nature*, *498*(7455), 502-505. https://doi.org/10.1038/nature12216

Udeinya, I. J. (1990). In vitro and ex-vivo models of sequestration in Plasmodium falciparum infection. *Am J Trop Med Hyg*, *43*(2 Pt 2), 2-5. https://doi.org/10.4269/ajtmh.1990.43.2

Udeinya, I. J., Miller, L. H., McGregor, I. A., & Jensen, J. B. (1983). Plasmodium falciparum strain-specific antibody blocks binding of infected erythrocytes to amelanotic melanoma cells. *Nature*, *303*(5916), 429-431. https://doi.org/10.1038/303429a0

Udeinya, I. J., Schmidt, J. A., Aikawa, M., Miller, L. H., & Green, I. (1981). Falciparum malaria-infected erythrocytes specifically bind to cultured human endothelial cells. *Science*, *213*(4507), 555-557. https://doi.org/10.1126/science.7017935

Udomsangpetch, R., Reinhardt, P. H., Schollaardt, T., Elliott, J. F., Kubes, P., & Ho, M. (1997). Promiscuity of clinical Plasmodium falciparum isolates for multiple adhesion molecules under flow conditions. *J Immunol*, *158*(9), 4358-4364.

Uwimana, A., Legrand, E., Stokes, B. H., Ndikumana, J. M., Warsame, M., Umulisa, N., Ngamije, D., Munyaneza, T., Mazarati, J. B., Munguti, K., Campagne, P., Criscuolo, A., Ariey, F., Murindahabi, M., Ringwald, P., Fidock, D. A., Mbituyumuremyi, A., & Menard, D. (2020). Emergence and clonal expansion of in vitro artemisinin-resistant Plasmodium falciparum kelch13 R561H mutant parasites in Rwanda. *Nat Med*, *26*(10), 1602-1608. https://doi.org/10.1038/s41591-020-1005-2

van den Berg, R. H., Prins, F., Faber-Krol, M. C., Lynch, N. J., Schwaeble, W., van Es, L. A., & Daha, M. R. (1997). Intracellular localization of the human receptor for the globular domains of C1q. *J Immunol*, *158*(8), 3909-3916.

van Leeuwen, H. C., & O'Hare, P. (2001). Retargeting of the mitochondrial protein p32/gC1Qr to a cytoplasmic compartment and the cell surface. *J Cell Sci*, *114*(Pt 11), 2115-2123. https://doi.org/10.1242/jcs.114.11.2115

Vigan-Womas, I., Guillotte, M., Juillerat, A., Hessel, A., Raynal, B., England, P., Cohen, J. H., Bertrand, O., Peyrard, T., Bentley, G. A., Lewit-Bentley, A., & Mercereau-Puijalon, O. (2012). Structural basis for the ABO blood-group dependence of Plasmodium falciparum rosetting. *PLoS Pathog*, *8*(7), e1002781. https://doi.org/10.1371/journal.ppat.1002781

Voss, T. S., Healer, J., Marty, A. J., Duffy, M. F., Thompson, J. K., Beeson, J. G., Reeder, J. C., Crabb, B. S., & Cowman, A. F. (2006). A var gene promoter controls allelic exclusion of virulence genes in Plasmodium falciparum malaria. *Nature*, *439*(7079), 1004-1008. https://doi.org/10.1038/nature04407

Waitumbi, J. N., Opollo, M. O., Muga, R. O., Misore, A. O., & Stoute, J. A. (2000). Red cell surface changes and erythrophagocytosis in children with severe Plasmodium falciparum anemia. *Blood*, *95*(4), 1481-1486. https://doi.org/10.1182/blood.V95.4.1481.004k15_1481_1486

Wang, Y., Finan, J. E., Middeldorp, J. M., & Hayward, S. D. (1997). P32/TAP, a cellular protein that interacts with EBNA-1 of Epstein-Barr virus. *Virology*, *236*(1), 18-29. https://doi.org/10.1006/viro.1997.8739

Warimwe, G. M., Fegan, G., Musyoki, J. N., Newton, C. R., Opiyo, M., Githinji, G., Andisi, C., Menza, F., Kitsao, B., Marsh, K., & Bull, P. C. (2012). Prognostic indicators of life-threatening malaria are associated with distinct parasite variant antigen profiles. *Sci Transl Med*, *4*(129), 129ra145. https://doi.org/10.1126/scitranslmed.3003247

Warrell, D. A., White, N. J., Veall, N., Looareesuwan, S., Chanthavanich, P., Phillips, R. E., Karbwang, J., Pongpaew, P., & Krishna, S. (1988). Cerebral anaerobic glycolysis and reduced cerebral oxygen transport in human cerebral malaria. *Lancet*, *2*(8610), 534-538. https://doi.org/10.1016/s0140-6736(88)92658-x

Wassmer, S. C., Combes, V., Candal, F. J., Juhan-Vague, I., & Grau, G. E. (2006). Platelets potentiate brain endothelial alterations induced by Plasmodium falciparum. *Infect Immun*, *74*(1), 645-653. https://doi.org/10.1128/iai.74.1.645-653.2006

Wassmer, S. C., Lépolard, C., Traoré, B., Pouvelle, B., Gysin, J., & Grau, G. E. (2004). Platelets reorient Plasmodium falciparum-infected erythrocyte cytoadhesion to activated endothelial cells. *J Infect Dis*, *189*(2), 180-189. https://doi.org/10.1086/380761

Wassmer, S. C., Moxon, C. A., Taylor, T., Grau, G. E., Molyneux, M. E., & Craig, A. G. (2011). Vascular endothelial cells cultured from patients with cerebral or uncomplicated malaria exhibit differential reactivity to TNF. *Cell Microbiol*, *13*(2), 198-209. https://doi.org/10.1111/j.1462-5822.2010.01528.x

Wassmer, S. C., Taylor, T., Maclennan, C. A., Kanjala, M., Mukaka, M., Molyneux, M. E., & Grau, G. E. (2008). Platelet-induced clumping of Plasmodium falciparum-infected erythrocytes from Malawian patients with cerebral malaria-possible modulation in vivo by thrombocytopenia. *J Infect Dis*, *197*(1), 72-78. https://doi.org/10.1086/523761

Watson, J. A., Uyoga, S., Wanjiku, P., Makale, J., Nyutu, G. M., Mturi, N., George, E. C., Woodrow, C. J., Day, N. P. J., Bejon, P., Opoka, R. O., Dondorp, A. M., John, C. C., Maitland, K., Williams, T. N., & White, N. J. (2022). Improving the diagnosis of severe malaria in African children using platelet counts and plasma PfHRP2 concentrations. *Sci Transl Med*, *14*(654), eabn5040. https://doi.org/10.1126/scitranslmed.abn5040

Weiss, G. E., Gilson, P. R., Taechalertpaisarn, T., Tham, W. H., de Jong, N. W., Harvey, K. L., Fowkes, F. J., Barlow, P. N., Rayner, J. C., Wright, G. J., Cowman, A. F., & Crabb, B. S. (2015). Revealing the sequence and resulting cellular morphology of receptor-ligand interactions during Plasmodium falciparum invasion of erythrocytes. *PLoS Pathog*, *11*(2), e1004670. https://doi.org/10.1371/journal.ppat.1004670

Weksler, B., Romero, I. A., & Couraud, P. O. (2013). The hCMEC/D3 cell line as a model of the human blood brain barrier. *Fluids Barriers CNS*, *10*(1), 16. https://doi.org/10.1186/2045-8118-10-16

Weksler, B. B., Subileau, E. A., Perrière, N., Charneau, P., Holloway, K., Leveque, M., Tricoire-Leignel, H., Nicotra, A., Bourdoulous, S., Turowski, P., Male, D. K., Roux, F., Greenwood, J., Romero, I. A., & Couraud, P. O. (2005). Blood-brain barrier-specific properties of a human adult brain endothelial cell line. *Faseb j*, *19*(13), 1872-1874. https://doi.org/10.1096/fj.04-3458fje

White, N. J. (1996). The treatment of malaria. *N Engl J Med*, *335*(11), 800-806. https://doi.org/10.1056/nejm199609123351107

White, N. J. (2018). Anaemia and malaria. *Malar J*, *17*(1), 371. https://doi.org/10.1186/s12936-018-2509-9

WHO. (2000). Severe falciparum malaria. World Health Organization, Communicable Diseases Cluster. *Trans R Soc Trop Med Hyg*, *94 Suppl 1*, S1-90.

WHO. (2014). Severe malaria. *Trop Med Int Health*, *19 Suppl 1*, 7-131. https://doi.org/10.1111/tmi.12313_2

WHO. (2021). *WHO malaria terminology, 2021 update*. https://iris.who.int/bitstream/handle/10665/349442/9789240038400-eng.pdf

WHO. (2024). *World Malaria Report 2024: addressing inequity in the global malaria response*. https://iris.who.int/.

Wolburg, H., & Lippoldt, A. (2002). Tight junctions of the blood-brain barrier: development, composition and regulation. *Vascul Pharmacol*, *38*(6), 323-337. https://doi.org/10.1016/s1537-1891(02)00200-8

Woodruff, A. W., Ansdell, V. E., & Pettitt, L. E. (1979). Cause of anaemia in malaria. *Lancet*, *1*(8125), 1055-1057. https://doi.org/10.1016/s0140-6736(79)92952-0

Xiao, L., Yang, C., Dorovini-Zis, K., Tandon, N. N., Ades, E. W., Lal, A. A., & Udhayakumar, V. (1996). Plasmodium falciparum: involvement of additional receptors in the cytoadherence of infected erythrocytes to microvascular endothelial cells. *Exp Parasitol*, *84*(1), 42-55. https://doi.org/10.1006/expr.1996.0088

Yipp, B. G., Anand, S., Schollaardt, T., Patel, K. D., Looareesuwan, S., & Ho, M. (2000). Synergism of multiple adhesion molecules in mediating cytoadherence of Plasmodium falciparum–infected erythrocytes to microvascular endothelial cells under flow. *Blood*, *96*(6), 2292-2298. https://doi.org/10.1182/blood.V96.6.2292

Yipp, B. G., Hickey, M. J., Andonegui, G., Murray, A. G., Looareesuwan, S., Kubes, P., & Ho, M. (2007). Differential roles of CD36, ICAM-1, and P-selectin in Plasmodium falciparum cytoadherence in vivo. *Microcirculation*, *14*(6), 593-602. https://doi.org/10.1080/10739680701404705

Yu, L., Loewenstein, P. M., Zhang, Z., & Green, M. (1995). In vitro interaction of the human immunodeficiency virus type 1 Tat transactivator and the general transcription factor

TFIIB with the cellular protein TAP. *J Virol*, *69*(5), 3017-3023. https://doi.org/10.1128/jvi.69.5.3017-3023.1995

Zhang, X., Florini, F., Visone, J. E., Lionardi, I., Gross, M. R., Patel, V., & Deitsch, K. W. (2022). A coordinated transcriptional switching network mediates antigenic variation of human malaria parasites. *Elife*, *11*. https://doi.org/10.7554/eLife.83840

Zougbede, S., Miller, F., Ravassard, P., Rebollo, A., Ciceron, L., Couraud, P. O., Mazier, D., & Moreno, A. (2011). Metabolic acidosis induced by Plasmodium falciparum intraerythrocytic stages alters blood-brain barrier integrity. *J Cereb Blood Flow Metab*, *31*(2), 514-526. https://doi.org/10.1038/jcbfm.2010.121