COLLEGE OF BASIC AND APPLIED SCIENCES

SCHOOL OF BIOLOGICAL SCIENCES

THE ROLE OF AN INTERCELLULAR ADHESION MOLECULE 1 BINDING MOTIF IN PLASMODIUM FALCIPARUM ERYTHROCYTE MEMBRANE PROTEIN 1 AND OTHER DETERMINANTS INVOLVED IN SEVERE MALARIA PATHOGENESIS

BY

GERTRUDE ECKLU-MENSAH

(10051041)

THIS THESIS IS SUBMITTED TO THE DEPARTMENT OF BIOCHEMISTRY, CELL AND MOLECULAR BIOLOGY, UNIVERSITY OF GHANA, LEGON IN PARTIAL FULFILLMENT OF THE REQUIREMENT FOR THE AWARD OF DOCTOR OF PHILOSOPHY IN BIOCHEMISTRY DEGREE

JULY, 2017

DECLARATION

This thesis was carried out under the supervision of Dr. Michael F. Ofori, Professors Michael D. Wilson, Anja T.R. Jensen and Lars Hviid.

I hereby declare that this is the result of my own research work and that other people's work has been duly acknowledged by reference. I further declare that that no material presented in this work has been submitted to any other institution for acquiring a degree. The paper published in Cell Host and Microbe (Appendix E) was a collaborative study, in addition to researchers from the University of Ghana and the University of Copenhagen involved different partners from other institutions.

I outline below the various contributions made by partners to this study.

Method/analysis	**Responsibility**
Sample collection, processing and storage	shared
Bioinformatics	partner
BOEC culturing	sole and shared
Crystal- and SPR work	partner
ELISA	sole and shared
Expression and purification of recombinant proteins	sole and shared
Flow cytometry	sole and shared
Immunofluorescence assay	partner
Parasite adhesion assays	sole and shared
Parasite culturing	sole and shared
Parasite selection	sole and shared
Real-time Q-PCR	sole and shared
Thesis preparation	sole

……

GERTRUDE ECKLU-MENSAH
(CANDIDATE)

DATE…….

……

DR MICHAEL FOKUO OFORI

(PRINCIPAL SUPERVISOR)

DATE…….

……

PROFESSOR MICHAEL DAVID WILSON
(SUPERVISOR)

DATE……..

……

PROFESSOR ANJA TATIANA RAMSTEDT JENSEN
(SUPERVISOR)

DATE……

ABSTRACT

A central pathogenic feature in *Plasmodium falciparum* cerebral malaria (CM) is the sequestration of infected erythrocytes (IEs) which results in the obstruction of blood flow, inflammation and damage to the endothelial cell barrier integrity. Adhesion is mediated by a family of approximately 60 highly variant parasite proteins called *P. falciparum* erythrocyte membrane protein 1 (PfEMP1), which are displayed on the surface of infected cells. All PfEMP1 contain multiple extracellular domains of Duffy binding like (DBL) and cysteine-rich interdomain region (CIDR) proteins that mediate adhesion to various host endothelial receptors such as intercellular adhesion molecule 1 (ICAM-1). An important unanswered question in malaria pathogenesis has always been whether a specific binding phenotype can be linked to any of the different severe malaria phenotypes. The broad objectives of this study were to investigate the involvement of an ICAM-1 binding motif in PfEMP1 in the development of CM, as well as to evaluate the contributions of the immune response and other determinants driving severe malaria complications.

A highly conserved motif in DBLβ domain of group A PfEMP1 molecules was identified using sequence information. IEs expressing PfEMP1 containing the motif had a dual binding affinity for endothelial protein C receptor (EPCR) and ICAM-1 when tested under *in vitro* flow adhesion assays. Quantitative real time PCR (qPCR) showed higher transcription of this motif in parasites isolated from children with CM compared with non-cerebral severe malaria ($p=0.020$). The sequences defining the motif were exceptionally conserved and thus suggested its ability to induce cross-reactive responses. Broadly reactive cross-inhibitory antibodies against the motif were acquired following *P. falciparum* natural infections, in addition to being experimentally induced. Such cross-reactive antibodies interfered with ICAM-1

binding of recombinant motif-containing DBLβ domains and IEs from paediatric CM patients. These findings make the motif an attractive candidate to be included in a vaccine cocktail that seeks to prevent CM complications.

Markers of endothelial activation (sICAM-1, sEPCR, Ang-2, HMGB1) in Ghanaian children with severe or uncomplicated malaria and their levels following resolution of infection were determined using ELISA. The levels were higher in malaria patients in comparison with healthy controls ($p < 0.001$). These levels declined significantly in convalescent patients except for high mobility group box 1 protein (HMGB1) which remained high to a similar degree as observed during infection ($p > 0.05$), an observation reported for the first time in malaria.

Blood outgrowth endothelial cells (BOECs) from peripheral blood mononuclear cells of Ghanaian malaria patients were isolated and characterised as physiologically relevant cells to potentially study *falciparum*-infected erythrocyte adhesive interactions. Flow cytometry analysis showed that BOECs expressed several endothelial surface markers including malaria parasite adhesion proteins ICAM-1 and EPCR. Thus, future use of these cells could advance our understanding of cytoadhesion involving both receptors.

Very little is known about the frequency of IgM-binding PfEMP1 in the *P. falciparum* genome, a phenotype that has been associated with severe disease. Quantitative real time PCR was used to assess the *var* transcript levels of 3D7/NF54 parasites selected for IgM binding. This study uncovered at least six new *var*/PfEMP1 candidates that could potentially mediate binding to IgM.

Overall, this study expands current knowledge on the contributions made by both host and parasite factors to severe malaria pathogenesis. Detailed knowledge of these factors is crucially important for effective management of malaria.

DEDICATION

To all children under five years of age in malaria endemic countries

ACKNOWLEDGEMENTS

First to my supervisors, Dr. Michael Ofori, Professors Michael Wilson, Anja Jensen and Lars Hviid for your valuable support and guidance throughout the PhD journey. I am highly indebted to Anja Jensen who welcomed me with both arms into her group and has been a great and patient mentor. Thank you for making me feel at home in Copenhagen, for the get-togethers and good motivation during trying moments. I am very grateful to Lars Hviid for keeping his doors wide open. I could always enter without prior notice and he was ever prepared to have a discussion. Thank you for accepting me as your scientific daughter, for your enthusiasm in my work and frank advice. My PhD study would have been very fruitless without the cooperation of the consented participants, parents and guardians in Hohoe. Thank you very much!

To the super smart Target ladies: Anja B, Mette, Rebecca, Soffie, Vanessa and Yvonne, I am very grateful for the contributions made to my project, for good times in and outside the lab. Thanks to Yvonne for introduction into the world of endothelial cells, Anja B for teaching me cytoadhesion assays, Vanessa, for a memorable eight month period in and outside the lab. I do share my award from the Danish Society for Parasitologists spring symposium with you. Thanks to past and present members of the Surface team, for great friendship and amazing collaboration. My sincerest thanks to Pilar and Mary for sharing their rich experiences with me. My gratitude goes to the staff at the CMP, University of Copenhagen for the interactive and multinational environment and even more for the stimulating discussions at scientific updates. Many thanks to Christian and Regis, for giving me a long lasting impression about wonderful Copenhagen and your delightful personalities. Regis, I am very grateful for the timely support. Thanks to the cake club members for divine cakes. My sincerest appreciation to my MAVARECA colleagues: William, Frederica and Betty for the

scientific discussions and sharing in my many cheers and wails. I highly acknowledge contributions made by the Director of Noguchi Memorial Institute for Medical Research, Prof. Kwadwo Koram and also kind contributions from the Immunology Department particularly Rev. Alex Danso, Kakra Dickson, Eric Kyei-Baffour, Ben Tornyigah and Cecilia Smith. A special thanks to the staff of the Hohoe Municipal Hospital and the Onchocerciasis unit for good collaboration and field support throughout my project. I am thankful to the staff of Biochemistry, Cell and Molecular Biology Department, University of Ghana for their support and valuable advice. I am very grateful to Anja B, Mary, Pilar, Samira, William vanP. and Yvonne for proof reading and constructive comments on my work. Samira and Mary, your useful insights and efforts are highly acknowledged mucho gracias!

Special thanks to two smart colleagues Isaac Darko and Sam Aboagye for the lively scientific and social discussions and more importantly for not allowing me to sink into the PhD ocean. Thanks to very loyal friends: Andy, Chantal, Henrietta, Jose P, Simone, Sylvia, Lily, Lydia, Phinna, Richard Appiah Otoo for always believing in me. I am truly humbled. Special thanks to Frs. Ben Ohene, Dominic Ofori and Andrej for your constant prayers and motivation. Fr. Ofori, your frequent calls and rich experiences have been a wonderful blessing. My kindest appreciation to Drs. A. Ghansah and B. Egyir for the incredible advice and encouragement. I am deeply grateful to Augustine Awoyale for your extraordinary support and being my number one enthusiastic cheer leader. To my parents and siblings, for the numerous sacrifices, patience, prayers and unconditional support, thank you is not enough! I owe a lot of gratitude to the Danish ministry of foreign affairs (DANIDA) for generously funding my PhD study.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

Abs	Antibodies
ACT	Artemisinin-based combination therapy
Ang	Angiopoietin
ANOVA	Analysis of variance
APC	Activated protein C
BCS	Blantyre coma score
BOEC	Blood outgrowth endothelial cells
BSA	Bovine serum albumin
BSD	Blasticidin S deaminase
CC	Community controls
CD	Cluster of differentiation
cDNA	Complementary deoxyribonucleic acid
CHO	Chinese hamster ovary
CIDR	Cysteine-rich interdomain region
CM	Cerebral malaria
CSA	Chondroitin sulphate A
Ct	Cycle threshold
DAPI	4',6- diamidino-2-phenylindole dihydrochloride
DBL	Duffy-binding like
DC	Domain cassette
DEPC	Diethylpyrocarbonate
DPBS	Dulbecco's phosphate buffered saline
E. coli	*Escherichia coli*
EC	Endothelial cell
ECFC	Endothelial colony forming cells
EDTA	Ethylenediaminetetracetic acid
EGM-2	Endothelial cell growth medium-2

ELISA	Enzyme -inked immunosorbent assay
EPCR	Endothelial protein C receptor
FBS	Foetal bovine serum
2 % FBS	2 % foetal bovine serum in phosphate buffered saline
Fc	Fragment crystallisable (tail) region of an immunoglobulin
FC	Febrile controls
FITC	Fluorescein isothiocyanate
GLURP	Glutamate-rich protein
GLURP-R2	C-terminal repeat region-2 of GLURP
H	Hour (s)
H_2O	Water
H_2O_2	Hydrogen peroxide
HRP	Horse radish peroxidase
H_2SO_4	Sulphuric acid
H3K4me2/3	Histone 3 lysine 4 bi and tri-methylation
Hb	Haemoglobin
HBEC5i	Human brain endothelial cells
HBMEC	Human brain microvascular endothelial Cells
HCl	Hydrochloric acid
HDMEC	Human eermal microvascular endothelial cells
HEK	Human embryonic kidney
His	Histidine
HMGB1	High mobility group box 1
ICAM-1	Intercellular adhesion molecule-1
^{125}I	Iodine-125
IE	Infected erythrocytes
IFNγ	Interferon gamma
IgG	Immunoglobulin G
IgG-HRP	Horseradish peroxidase conjugated to Immunoglobulin G

IgM	Immunoglobulin M
IL	Interleukin
IPTp	Intermittent preventive therapy in pregnancy
LFA-1	Lymphocyte function-associated antigen-1
LLIN	Long-lasting insecticide-treated nets
Mac1	Macrophage-1 antigen
MACS	Magnetic activated cell sorting
MFI	Mean fluorescent intensity
Min	Minute
MOH	Ministry of Health
MSP2	Merozoite surface protein-2
NaCl	Sodium chloride
NTS	N-terminal segment
OD	Optical density
OM	Other malaria
OPD	Out-patients department
PAM	Pregnancy-associated malaria
PAR1	Protease-activated receptor 1
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PECAM-1	Platelet endothelial cell adhesion molecule 1
PfEMP1	*Plasmodium falciparum* erythrocyte membrane protein 1
PfHP1	*P. falciparum* heterochromatin protein 1
PfRNAse II	*P. falciparum* exoribonuclease II
PfSETvs	*P. falciparum* variant-silencing SET gene
qPCR	Quantitative real-time polymerase chain reaction
rCD36	Recombinant cluster of differentiation 36
rEPCR	Recombinant endothelial protein C receptor
rICAM-1	Recombinant intercellular adhesion molecule-1

RDT	Rapid diagnostic test
RIF/RIFIN	Repetitive interspersed family
RNA	Ribonucleic acid
pol II	Polymerase enzyme II
RPMI	Roswell Park Memorial Institute
S	Second (s)
SA	Severe anaemia
SD	Standard deviation
SEM	Standard error of mean
sEPCR	soluble Endothelial protein C receptor
sICAM-1	soluble Intercellular adhesion molecule-1
SIR2	Silent Information Regulator 2
SM	Severe malaria
STEVOR	Sub-telomeric variable open reading frame
Temp	Temperature
TM	Thrombomodulin
TNF-α	Tumour necrosis factor alpha
Tris	Tris(hydroxymethyl)aminomethane
Tu	Transcript units
UK	United Kingdom
UM	Uncomplicated malaria
Ups	Upstream promoter sequence
USA	United States of America
VSA	Variant surface antigens `
vWF	von Willebrand factor
WHO	World Health Organisation
Wk	Week

CHAPTER ONE

INTRODUCTION

1.1 Background

Plasmodium falciparum causing malaria is largely responsible for most childhood malaria-related deaths and morbidity. Severe *falciparum* malaria manifests as three overlapping syndromes of severe anaemia (SA), cerebral malaria (CM) and respiratory distress. By far, CM is the most lethal disease outcome with a fatality rate of about 20 % even after adherence to effective anti-malarial treatments (Birbeck *et al*., 2010). A minority of CM survivours are also known to sustain variable degrees of neurocognitive disorders (reviewed in Hviid and Jensen, 2015; Birbeck *et al*., 2010). The mechanisms underlying the development of CM remain poorly elucidated but are believed to involve cytoadhesion of the *P. falciparum* virulent protein, *Plasmodium falciparum* erythrocyte membrane protein 1 (PfEMP1) to human endothelial receptors such as intercellular adhesion molecule-1 (ICAM-1) and endothelial protein C receptor (EPCR), alterations in inflammatory cytokines and endothelial activation (reviewed in Storm and Craig, 2014; Cunnington *et al*., 2013b).

The general objectives of this study were to investigate the role of an ICAM-1 binding motif in PfEMP1, in the development of CM, as well as evaluate the contributions of the immune response and other determinants involved in severe disease.

1.2 Objective 1: The involvement of an ICAM-1 binding PfEMP-1 motif in CM

Cytoadhesion of infected erythrocytes (IEs) to receptors on the host endothelium is a prominently recognised pathological feature in CM development. The parasite protein thought to facilitate this adhesion process is PfEMP1, which is coded by *var* genes in a mutually exclusive order, resulting in the exposure of one or few PfEMP1 molecules at a time. Their expression can be altered at any given time to avoid immune recognition and enhance parasite survival (reviewed in Hviid and Jensen, 2015). PfEMP1 variants are generally classified into groups A-E and two hybrid groups (B/A, B/C) depending on their chromosomal location or into domain cassettes (DC) which involve particular arrangements of the adhesion domains of Duffy-binding like proteins (DBL) and cysteine-rich interdomain region proteins [CIDR] (Rask *et al*., 2010; Lavstsen *et al*., 2003). There is strong evidence that links the expression of group A and group B/A PfEMP1, including those that contain DC4, DC8 and DC13 to SM, while group C PfEMP1 is largely confined to uncomplicated malaria [UM] (Bengtsson *et al*., 2013b; Lavstsen *et al*., 2012; Rottmann *et al*., 2006; Jensen *et al*., 2004). With respect to the human host, a variety of receptors are well known to interact with PfEMP1, although there is no clear-cut evidence of the involvement of particular receptor(s) in CM.

ICAM-1 expressed on endothelial cells in the microvasculature is believed to serve as a receptor for adhesion of IEs during CM pathogenesis. Immunopathological evaluations of autopsy samples of CM patients reported increased endothelial ICAM-1 expression together with retained IEs in the brain microvasculature (Armah *et al*., 2005; Turner *et al*., 1998; Turner *et al*., 1994). However, the role of ICAM-1 in CM has not always been consistent. Notably, IEs that bind to ICAM-1 express DBLβ domains of groups B and C PfEMP1 that can also mediate CD36 binding which are

associated with UM; in addition, group A PfEMP1 expressing IEs linked to CM are also known to bind to ICAM-1. Moreover, IEs selected on human brain endothelial cells expressing PfEMP1 of DC8 and DC13 do not bind ICAM-1 but EPCR through their CIDRα domain (Turner *et al*., 2013). Altogether suggesting that, group A PfEMP1 involved in CM may not preferentially bind ICAM-1 providing a weak evidence for the role of ICAM-1 in the development of CM.

Thus Bengtsson *et al* (2013) sought to clarify the role of group A PfEMP1 ICAM-1 binding in CM. The authors identified a group of 3D7 group A PfEMP1 PFD1235w (PlasmoDB: PF3D7_0425800) orthologous proteins in parasite isolates from *falciparum*-infected Ghanaian children. This group of proteins possessed a highly similar N-terminal three tandem domain (DBLα1.1/1.4-CIDRα1.6- DBLβ3) which was identified as domain cassette 4 (DC4). IEs selected to express DC4 PfEMP1 adhered to ICAM-1 and not CD36, a phenotype linked to UM and the binding capacity mapped to the C-terminal portion of the DBLβ3 domain. Indeed, DBLβ3 domain-specific antibodies acquired during natural infections interfered with ICAM-1 binding as well as cross-reactive of ICAM-1 binding across different parasite genomes containing DC4. Further experiments resulted in the characterisation of a conserved ICAM-1 binding motif within the DBLβ3 region. The CIDRα subtype in DC4 was also found to be present in the EPCR binding PfEMP1 of DC13, suggesting the potential of IEs with such domains to bind two different endothelial receptors. Although Bengtsson *et al* (2013b) used laboratory-adapted parasite lines and very few field isolates, the findings demonstrated that parasites harbouring this ICAM-1 binding motif may be relevant for vaccine development that inhibits malaria parasite sequestration in the brain. Therefore, it is imperative to investigate the widespread occurrence of parasites expressing PfEMP1 containing the ICAM-1 binding motif

predicted to cause CM in field isolates, demonstrate their ability to bind to ICAM-1 and link this binding to clinical disease. In addition, it is important to show the ability of motif-specific antibodies to inhibit ICAM-1 binding, which offers clinical protection. The aim of this study was to dissect further the molecular details characterising ICAM-1 binding by group A PfEMP1 by using sequence information to define a sequence motif that can be used to identify ICAM-1-binding DBLβ domains. Additionally, the relationship between the expression of DBLβ domains involved in ICAM-1 binding and CM development was investigated.

Hypothesis 1: *Plasmodium falciparum* IEs associated with CM express a particular subset of group A PfEMP1 that bind to both ICAM-1 and EPCR and contain specific amino acid sequences (motif) that mediates the binding.

Specific objective 1.1: To assess the transcription levels of motif-encoding *var* genes and EPCR binding CIDRα domains in parasites obtained from defined categories of malaria patients.

Specific objective 1.2: To assess the binding of *P. falciparum* IE to ICAM-1 and EPCR under physiological flow conditions

Specific objective 1.3: To measure motif-specific IgG in plasma of children with acute malaria.

1.3 Objective 2: The involvement of host immune responses in malaria

There is overwhelming data showing that a robust immune response is evoked during *P. falciparum* infection. The immune response involves a delicate compromise between pro- and anti-inflammatory cytokines which are important for the control of parasite multiplication and ultimate clearance of infection (reviewed in Crompton *et al*., 2014). The collapse of this balance has been consistently linked to the events that characterise SM pathogenesis. Indeed many studies report elevated levels of pro-inflammatory cytokines in children with SM relative to UM patients or an increase in the proportion of pro- to anti-inflammatory cytokines (Dodoo *et al*., 2002; Kurtzhals *et al*., 1998; Kwiatkowski *et al*., 1990). The joint actions of parasite sequestration and excessive host inflammatory response are involved in the self-amplification of SM pathology resulting from widespread activation of the endothelium and release of activation markers. Endothelial activation and inflammation result in the upregulation of constitutively expressed endothelial ligands, which in-turn promotes massive sequestration leading to a compromise in the vascular integrity (Abdi *et al*., 2014). In this study, biomarkers of inflammation and endothelial activation were measured in paediatric malaria patients and their levels following disease resolution were evaluated.

Hypothesis 2: Higher levels of endothelial markers of activation are associated with SM

Specific objective 2.1: To assess plasma for biomarkers of activation and inflammation in acute and convalescent children with either UM or SM.

1.4 Objective 3: Establishment of a blood outgrowth endothelial cell *in vitro* system and its potential use to study *P. falciparum* cytoadhesion properties

Studies on the interactions between malaria parasites and receptors on the host endothelium have largely relied on postmortem samples, murine or *in vitro* models of non-human origin. Although these studies have provided incredible insights, they have peculiar challenges (reviewed in White *et al*., 2010; Andrews *et al*., 2005). Thus, there is an ever-growing interest to establish more *in vitro* models to unravel the molecular details of cytoadhesion which may offer anti-adhesion therapies to complement existing treatment. Blood outgrowth endothelial cells (BOECs) represent an alternate *in vitro* tool to study parasite binding characteristics since it has the capacity to express diverse malaria parasite adhesion receptors. Moreover, they are an easily accessible source of material present in peripheral blood mononuclear cells (PBMCs) and are culture adaptable with a very high proliferative potential (Lin *et al*., 2000). They can be derived from malaria patients and hence serve as physiologically relevant cells to study parasite adhesion obtained from patients.

Hypothesis 3: Cultures of blood outgrowth endothelial cells (BOEC) can be established from small volumes of blood from children.

Specific objective 3.1: To isolate and characterise BOECs obtained from PBMCs of malaria patients.

1.5 Objective 4: Identification of PfEMP1 mediated IgM binding variants in *P. falciparum* genome

Apart from mediating adhesion to endothelial receptors, PfEMP1 variants are known to interact with components found in human serum, particularly non-immune IgM. This particular interaction has been observed in some IEs involved in rosetting and IEs binding to chondroitin sulphate A (CSA) in pregnancy-associated malaria (PAM), both phenotypes being linked with SM development (Creasey *et al*., 2003; Rowe *et al*., 2002). Although the physiological relevance of IgM binding is not well understood, it is believed that this interaction together with other serum factors reinforces the relatively weak interactions between IEs expressing PfEMP1 and their receptors on erythrocytes, thereby promoting disease severity (reviewed in Pleass *et al*., 2016). Recent data show that multiple PfEMP1 variants in a given parasite genome have the capacity to bind IgM. Indeed, Jeppesen *et al* (2015) provided evidence of at least five IgM-binding PfEMP1 subtypes in the genome of *P. falciparum* NF54. There remains a possibility of identifying more IgM-binding PfEMP1 within a parasite genome and it is pertinent to investigate this given the clinical importance of IgM binding.

Hypothesis 4: The *P. falciparum* NF54/3D7 genome encodes several uncharacterised IgM-binding PfEMP1 variants.

Specific objective 4.1: To identify PfEMP1 variants involved in IgM binding in the NF54/3D7 parasite.

CHAPTER TWO

LITERATURE REVIEW

2.1 Global burden and strategies

Malaria burden is difficult to estimate, notably in low-income countries where data collection and reporting quality are significant challenges. Nevertheless, the World Health Organisation (WHO) reported that about 3.2 billion people in 91 countries worldwide were in danger of being infected with malaria parasites while 1.2 billion of them were at high risk of developing malaria in the year 2015 (WHO, 2016). Approximately 200 million people were infected with malaria parasites globally, of which about 429,000 of them died. The vast majority of deaths (92 %) occurred in Sub-Saharan Africa, with the heaviest toll being children under the age of five and pregnant women (WHO, 2016), portraying malaria as one of the world's greatest human scourge.

Despite these alarming figures, incredible efforts have been made to reduce the global burden of malaria and this has resulted in an approximately 21 % significant reduction in malaria cases between 2010 and 2015 (WHO, 2016). In Africa alone, the number of malaria-infected cases in children between two and 10 years old has reduced by approximately 50 %, from 2000 to 2015 (Bhatt *et al*., 2015; Figure 2.1). These interventions have included rapid diagnostic tests (RDT), artemisinin-based combination therapy (ACT), intermittent preventative treatment (IPT) and long-lasting insecticide-treated bed nets (LLIN) (reviewed in Alonso and Tanner, 2013; Flaxman *et al*., 2010). More recently, the WHO has introduced a new initiative, entitled "T3: Test, Treat and Track" a programme which entreats malaria endemic countries to provide widespread access to diagnostic testing and treat people only after they have

been confirmed malaria positive in order to establish a robust monitoring and control system (Bhutta *et al*., 2014).

Although evidence shows that the implementation of these interventions are effective and proving successful, the parasites, as well as their mosquito hosts, have developed formidable strategies to thwart malaria control efforts. For instance, drug resistance to artemisinin, the only potent antimalarial available has emerged in the Greater Mekong region in Southeast Asia (Dondorp *et al*., 2009; Noedl *et al*., 2008), with significant threats to India (Tun *et al*., 2015); with most researchers concerned that it might spread to Africa and other endemic countries soon. Meanwhile, the transmitting *Anopheles* mosquitoes have developed resistance to pyrethroid insecticides (Knox *et al*., 2014). In addition, a switch in mosquito behaviour has been reported in Tanzania where mosquitoes have altered their feeding habits from night to day, smartly evading the protection offered by insecticide-treated bed nets (Russell *et al*., 2011). Indeed, the overwhelming burden caused by malaria for individuals and governments of endemic countries cannot be overemphasized as the disease is closely linked to hampered socio-economic development and poverty. Some notable factors accounting for lower rates of economic growth include effects of the disease on fertility, population growth, worker productivity, absenteeism, premature death and medical costs. Together, these factors worsen the plight of these endemic countries that are already considered as low income, further deepening their gap with developed countries (Nonvignon *et al*., 2016; Orem *et al*., 2012; Sachs and Malaney, 2002).

It is therefore obvious that additional tools such as more effective drugs and vaccines to complement existing interventions will remain vital for sustainable malaria control and a global health priority. Massive efforts have been made for several decades to develop a malaria vaccine but the promise of a highly effective vaccine has remained

elusive. The only vaccine, RTS,S which is yet to be deployed is only partially effective, conferring a fleeting protection against clinical malaria. Significantly, when trials were conducted in differing malaria transmission settings in Africa among children between 5-17 months, the vaccine reduced clinical malaria by about 39 %, with absolutely no protection conferred against SM among children who did not receive the fourth dosage of the vaccine (RTS,S, 2015). Thus, it does not represent the true 'gold standard' vaccine against malaria. Clearly, an in-depth understanding of the intricate immunity to malaria will undoubtedly be critical to the development of a vaccine, even if it offers protection against severe disease.

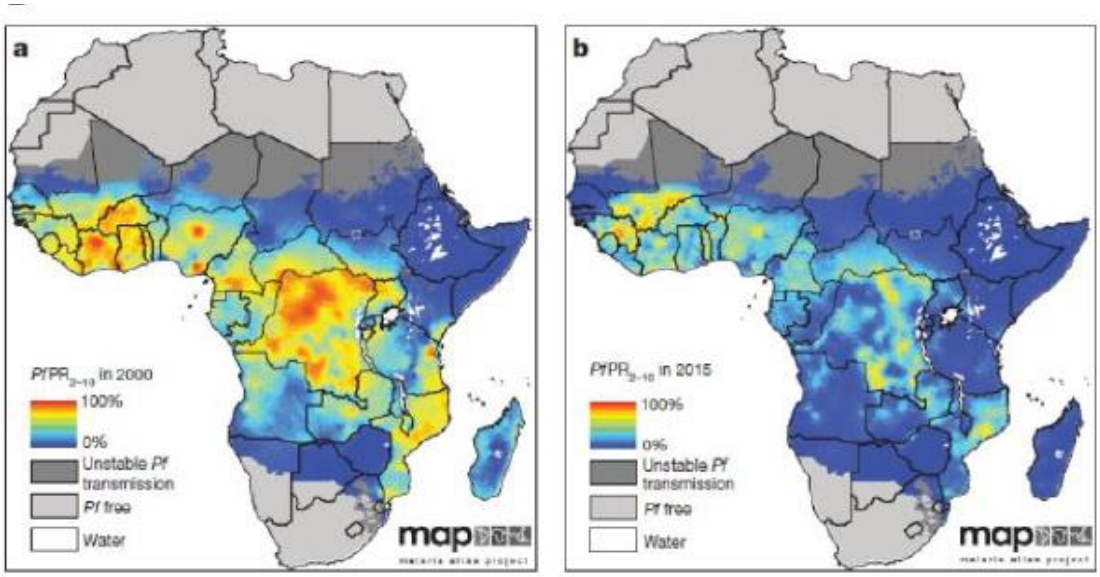


Figure 2.1: Changes in *P. falciparum* prevalence in 2-10 year olds in African countries. (a) The prevalence of *P. falciparum* in 2000 (b) *P. falciparum* prevalence in 2015. Gray regions represent *P. falciparum* free regions and blue zones areas with a low prevalence. Red spots are regions with the highest prevalence (Modified from Bhatt *et al*., 2015).

2.1.1 Malaria in Ghana

Ghana lies within the coastal belt of West Africa, bordered by Burkina Faso to the north, the Atlantic Ocean to the south, Cote d'Ivoire and Togo to the west and east respectively. In Ghana, everyone is at risk of getting infected with malaria parasites. The public health and economic burden of malaria in Ghana cannot be overemphasized as it remains one of the leading causes of morbidity and mortality. In 2013, malaria accounted for approximately 44 % of outpatient visits and 22.3 % of under-five mortality (MOH, 2014a). Reports show that it remains one of the most important causes of total work days lost and an estimated total cost of malaria to businesses is about US$ 6.5 million, with direct costs accounting for 90 % (Nonvignon *et al*., 2016). Malaria transmission in Ghana is intense, with peaks in the major wet season (April to July). *Plasmodium falciparum* is the predominant cause of malaria, transmitted by four major mosquito vectors: *Anopheles gambiae* (*sensu stricto*), *A. melas*, *A. arabiensis* and *A. funestus* (*sensu stricto*) (Duah *et al*., 2016). With regards to disease interventions, Ghana officially adopted the use of ACTs as the first choice of antimalarial drugs for the treatment of uncomplicated malaria in 2005 (Koram *et al*., 2005). Currently, the national malaria control programme of Ghana recommends artesunate–amodiaquine as the first line of treatment based on its efficacy, cost effectiveness and impact on the local industry, with artemether-lumefantrine and dihydroartemisinin-piperaquine as alternatives for those who cannot tolerate the reactions sometimes associated with artesunate–amodiaquine (Quashie *et al*., 2013; Koram *et al*., 2005). Complementary vector control strategies include LLINs and indoor residual spraying (MOH, 2014b).

2.2 Malaria parasites

Malaria is an infectious disease caused by the eukaryotic intracellular protozoan parasite belonging the kingdom *Protista*, phylum *Apicomplexa*, class *Sporozoa*, order *Eucoccidia* and genus *Plasmodium*. *Plasmodium* contains three genomes: a 23 Mb nuclear genome made up of 14 linear chromosomes, a very small linear mitochondrial genome and a 35 kb circular genome of red-algal ancestry found in the apicoplast (reviewed in Antinori *et al*., 2012). Sequence data reveal about 5,365 genes present on the 14 chromosomes, of which known functions have been assigned to approximately 1,800 genes (Gardner *et al*., 2002). Over 100 *Plasmodium* species infecting a diverse range of definitive hosts including reptiles, birds and mammals have been identified and out of these, four distinct parasite species are well adapted and known to traditionally infect humans: *Plasmodium falciparum, P. malariae*, *P. ovale* and *P. vivax* (reviewed in Miller *et al*., 2002). Either by natural or accidental means human beings occasionally can be infected with parasites from simian species such as *P. knowlesi* and *P. cynomolgi*. *Plasmodium knowlesi* has however emerged as an important cause of human malaria in South-East Asia and has therefore been considered a fifth human malaria parasite (reviewed in Antinori *et al*., 2012).

The current geographical distribution of *Plasmodium* species infecting humans shows *P. falciparum* as being endemic in tropical Africa, while *P. vivax* is more prevalent in South America compared to *P. falciparum* infections. Both *P. falciparum* and *P. vivax* are predominant in south-eastern Asia and western Pacific. *Plasmodium malariae* may occur in all malaria endemic areas however, its distribution is generally low. Mixed infections of *P. falciparum* and *P. malariae* are sometimes reported in the tropics of Africa. *Plasmodium ovale* is also infrequently observed in tropical Africa while *P. knowlesi* infection is concentrated in some forest zones of South-East Asia (reviewed

in Autino *et al*., 2012). *Plasmodium falciparum* remains the most lethal of all the infecting species accounting for more than 90 % of the world's malaria mortality (WHO, 2016; Snow *et al*., 2005) as such, most research has obviously focused more on *P. falciparum*. In recent times, *P. vivax* research has attracted a lot of interest largely due to its widespread geographic nature, and also increasing awareness of its involvement in severe disease and death which was previously thought to be benign (Naing *et al*., 2014; Baird, 2013).

2.3 Life cycle of human *Plasmodium* parasites

Malaria is transmitted from person to person through the bite of infected female Anopheline mosquitoes. There are over 435 identified species of *Anopheles* mosquitoes, of which 30-40 are vectors of major importance transmitting malaria parasites. In sub-Saharan Africa, *Anopheles gambiae, A. funestus* and *A. arabiensis* predominantly transmit malaria (reviewed in Cholewinski *et al*., 2015; Sinka *et al*., 2010). The human malaria parasite naturally inhabits two different hosts for successful completion of its life cycle. One-half of the cycle takes place in the female *Anopheles* mosquito, depicted by a sexual multiplication phase (sporogony) while the other half, characterised by an asexual phase (schizogony) occurs in the mammalian host (reviewed in Antinori *et al*., 2012). A schematic representation of the life cycle is shown in figure 2.2.

Human infection begins when *Plasmodium* sporozoites resident in the salivary gland of the infected *Anopheles* mosquito are injected into the skin of the human host along with anticoagulant-containing saliva to facilitate ingestion of blood. A proportion of sporozoites deposited in the skin vigorously migrate until they encounter a blood

vessel and enter the blood circulation. They rapidly invade hepatocytes and reside in a parasitophorous vacuole. This marks the onset of the parasite's liver-stage (pre-erythrocytic schizogony) development, lasting between two and 16 days depending on the species and also usually asymptomatic in patients. Sporozoites differentiate into liver stage schizonts within the parasitophorous vacuole, followed by further growth and multiple asexual nuclei divisions into thousands of newly formed merozoites. In *P. falciparum*, thousands of daughter merozoites can be produced within a week in a hepatic schizont; by contrast, *P. vivax* and *P. ovale* parasites can persist for a long period in the liver as dormant hypnozoites without any clinical presentation, which can be reactivated after weeks or even years later to cause relapses by invading the blood stream (Siciliano and Alano, 2015; Mota and Rodriguez, 2008; Silvie *et al*., 2008; Amino *et al*., 2006). Once the hepatic schizonts burst, released merozoites in circulation initiate the erythrocytic stage by infecting erythrocytes which are the primary sites of infection in malaria. The duration of the erythrocytic stage varies, depending on the transmitted species; it takes 48 h for *P. falciparum*, *P. vivax* and *P. ovale*, 72 h for *P. malariae* and 24 h for *P. knowlesi* (White *et al*., 2014).

Asexual division commences with parasites resident in a parasitophorous vacuole in erythrocytes. They transform from the ring stage followed by a growth phase leading to the formation of a trophozoite. There are multiple rounds of nuclear divisions resulting in the formation of schizonts, the final stage of asexual development in the human host. When schizonts mature, they rupture the host erythrocyte, releasing merozoites that rapidly invade new erythrocytes in circulation, continuing the asexual life cycle. The cyclic invasion and synchronous lysis of erythrocytes result in the release of parasites and erythrocytic material into circulation which triggers the production of pro-inflammatory cytokines which are responsible for the majority of

the clinical manifestations observed in a malaria patient (Tuteja, 2007). A small proportion of the merozoites diverge from the schizogony pathway to an alternate route of sexual commitment. Thus, these merozoites differentiate to produce male (micro) and female (macro) gametocytes, an action triggered by poorly elucidated mechanisms. These sexual erythrocytic stages have no further activity within the human host and are essential for transmitting the infection to *Anopheles* mosquitoes. A mosquito taking a blood meal on an infected individual may ingest these gametocytes into its midgut, where macrogametocytes form macrogametes and exflagellation of microgametocytes produce microgametes. These gametes fuse to form a diploid zygote that further transforms into a motile ookinete and penetrates the cell wall in the midgut to develop into an oocyst. Several mitotic divisions within the oocyst produce many sporozoites and when the oocyst ruptures, the released sporozoites migrate and localise in the salivary glands waiting to be transmitted to another host during the next blood meal (Tuteja, 2007).

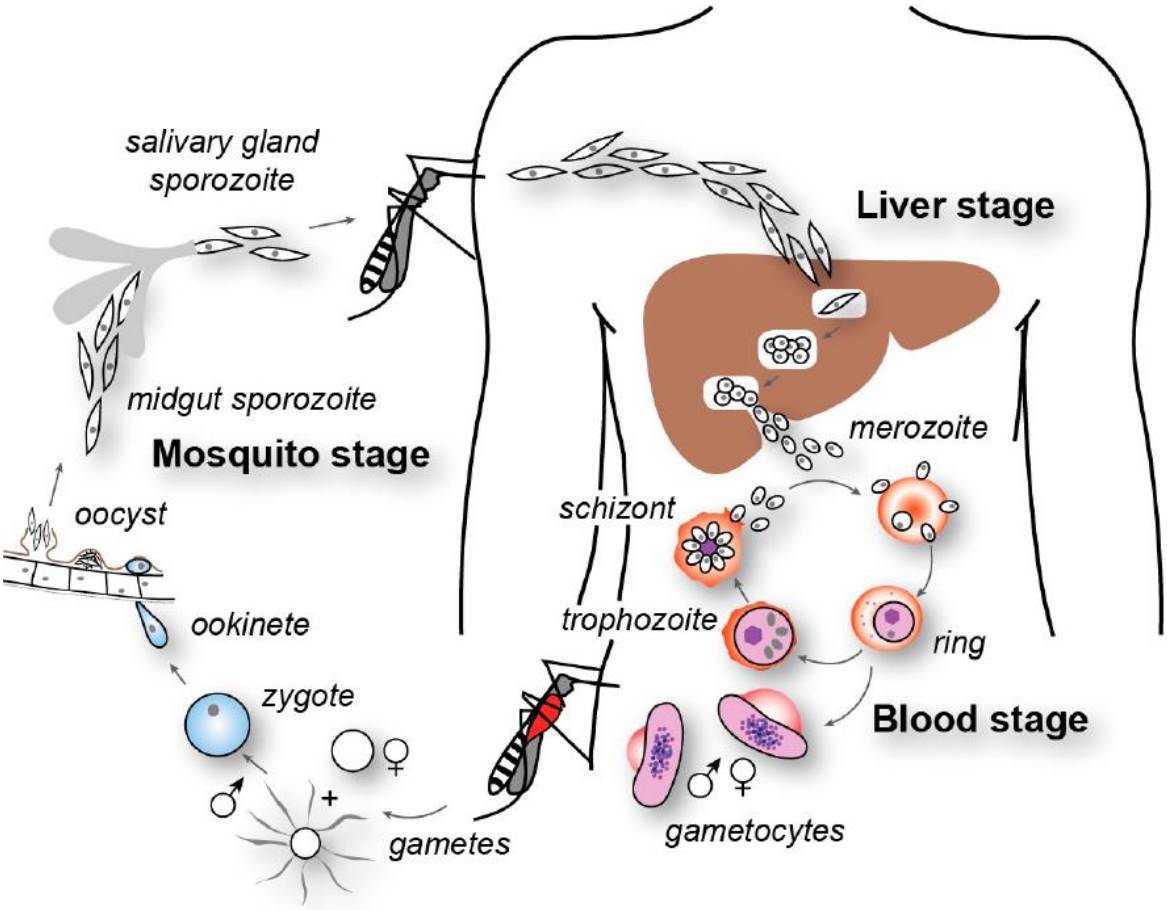


Figure 2.2: The life cycle of *P. falciparum*. The asexual part of the cycle occurs in humans and it involves a liver stage and a blood stage. The sexual stage occurs in the mosquito. Injected sporozoites from the *Anopheles* mosquito invade hepatocytes and develop into merozoites which are released into the bloodstream to invade erythrocytes. Development in the erythrocytes results in schizonts which are released to invade new erythrocytes. Some merozoites develop into sexual gametocytes which are picked up by the mosquito. Fusion of gametes forms a zygote which finally produces many sporozoites to continue the life cycle (Adapted from Cowman *et al*., 2012).

2.4 *Plasmodium falciparum* disease manifestations

The clinical presentations of malaria are diverse and overlapping, with a wide spectrum of disease severity which includes asymptomatic infection, mild, uncomplicated malaria and severe, life-threatening malaria. Clinical presentation and outcome appear to be determined by a vast number of factors including host immunity, virulence of the parasite, host age, as well as geographical and social factors (Crawley *et al*., 2010; Miller *et al*., 2002). Asymptomatic infection is highly prevalent and mostly associated with immune individuals. Such individuals have persistently low parasite densities with no clinical symptoms observed (Hamad *et al*., 2000). Disease pathogenesis is largely due to the multiplication of the asexual blood stage parasites since the liver stage is well known to be asymptomatic. Initial clinical signs of infection are nonspecific and typically mimic many of the diseases that are fever or flu related. They include fever, chills, headaches, nausea and malaise, which begin to appear about a week after inoculation of parasite sporozoites into the host. These symptoms generally coincide with the periodic lysis of infected erythrocytes containing matured parasites into circulation. These early classical symptoms are mostly observed in uncomplicated infections which is a common clinical syndrome in a majority of people and they usually resolve rapidly with the aid of a robust host immune system (reviewed in Storm and Craig, 2014).

However, a relatively few number of infections (about 1.0 %) if not timely treated do progress to severe, life-threatening disease as well as death through unexplained mechanisms (reviewed in Storm and Craig, 2014). SM (SM) is notably manifested in non-immune patients, young children from highly endemic locations, adults in low endemic regions and people with limited acquired immunity like primigravida (Storm and Craig, 2014; Hviid, 2010). Children under five years of age are most susceptible

to SM. Indeed, about 10-20 % of children are highly likely to suffer from severe malaria before the inception of acquired immunity in intense *P. falciparum* transmission areas and approximately 70 % of children may die due to malaria infection (WHO, 2016; Goncalves *et al*., 2014). It is a complex disease that may affect multiple organs and encompasses one or more of the following complications: SA, metabolic acidosis and CM, thus hampering exact diagnosis and disease classification (Goncalves *et al*., 2014; Storm and Craig, 2014; Idro *et al*., 2005; Mackintosh *et al*., 2004).

2.4.1 Severe anaemia

Severe malaria anaemia is largely responsible for most malaria-associated hospitalisations and deaths in children particularly from holoendemic transmission regions (Obonyo *et al*., 2007). It is further worsened by co-infections with bacteria, helminths and viruses which are very common in endemic regions, leading to high fatality particularly in children with secondary infections (Calis *et al*., 2008). The World Health Organisation (WHO) defines SA as having haemoglobin concentration < 5 g/dL (in children) partnered with *P. falciparum* parasites. Several factors are believed to contribute to the pathological processes of SA including the destruction of IEs accompanied by clearance of uninfected erythrocytes and decreased erythrocyte production (Awah *et al*., 2009; Lamikanra *et al*., 2007).

2.4.2 Metabolic acidosis

Metabolic acidosis and respiratory distress are often characterised by an unusual deep breathing and have been shown to be strong predictors in SM. Although the pathogenic mechanisms are not well defined, metabolic acidosis is enhanced by low oxygen levels in tissues as a consequence of anaemia, obstruction of microvascular blood flow caused by sequestration of IEs and rosetting. Accumulation of lactic acid resulting from the reduced clearance of the parasites by the liver has also been observed (reviewed in Marsh *et al*., 1996).

2.4.3 Cerebral malaria

CM represents one of the most lethal complications of malaria accounting for approximately 20 % of fatal cases in malaria endemic populations. CM is clinically defined as a state of unarousable coma with detectable *falciparum* parasites in peripheral blood and most of the times retinopathy, with the exemption of other potential causes of coma (Beare *et al*., 2011; Idro *et al*., 2005). Coma is assessed by a Blantyre score (1 or 2 out of 5) for children and Glasgow score (<11 on a scale of 15) for adults (Beare *et al*., 2011; Molyneux, 1989; Teasdale and Jennett, 1974).

CM can be treated and the recovery is relatively fast, however, some survivours are known to unfortunately suffer some residual brain-associated disabilities such as language disorder, motor deficits and cognitive loss (Birbeck *et al*., 2010) and there is no adjunctive therapy to aid their recovery. Although the molecular mechanisms leading to the development of CM and more precisely why some patients are able to recover while others suffer fatal outcomes remain difficult to explain, it is well recognised that the pathological processes differ between adults and children (Hawkes

et al., 2013; Dondorp, 2008). In addition, considerable insights from post-mortem observations reveal sequestered parasites localised in brain cells with wide-spread vascular pathology and disruption of blood brain barrier function (Dorovini-Zis *et al*., 2011). Consistent with that, CM in children has been placed in two subclasses based on the observed histopathological data. Children are classified as belonging to subgroup 1 (CM1) if they have sequestered parasites only while subgroup 2 (CM2) have sequestered parasites in addition to perivascular ring haemorrhages, fibrin platelet deposits and monocytes (Milner, Jr. *et al*., 2014; Moxon *et al*., 2013; Dorovini-Zis *et al*., 2011; Seydel *et al*., 2006). It is well acknowledged that a cocktail of factors involving the parasite and host drive CM pathogenesis (detailed in section 2.9).

2.4.4 Pregnancy-associated malaria, PAM

Pregnant women notably primigravids from endemic countries become vulnerable to severe *falciparum* malaria irrespective of their previous exposure. IEs adhere to CSA on syncytiotrophoblasts sequestering them in the placenta, which restricts blood flow as well as nutrients to the growing foetus. Also, excessive parasite sequestration stimulates the activation of host immune responses leading to placental inflammation (Conroy *et al*., 2013; Khattab *et al*., 2013; Suguitan, Jr. *et al*., 2003)**.** PAM results in increased risk of maternal anaemia, newborn anaemia, low birth weight, still-birth and pre-term delivery (Steketee *et al*., 2001).

2.5 Naturally-acquired immunity

After several years of encountering the parasites repeatedly, it is observed that there is an age-related decline in febrile malaria and parasite density in individuals living in endemic regions (Figure 2.3). This acquired immunity is variable depending on the transmission dynamics as it has been observed that residents in hyperendemic regions acquire clinical immunity relatively faster than people living in low transmission regions (reviewed in Fowkes *et al*., 2016; Langhorne *et al*., 2008). Generally, the building of natural immunity is a slow or gradual process which passes through different phases with immunity achieved relatively quicker against severe and deadly malaria. Children in the first few months of their life rarely suffer from severe forms of malaria disease as it is widely believed that they benefit from maternally protective antibodies (reviewed in Hviid, 2005). Conversely, severe disease is confined to toddlers generally beyond six months with immunity to non-cerebral severe disease rapidly acquired after one or two clinical episodes and complete immunity to severe disease and death attained generally by age of five years notably in areas of high malaria transmission (Griffin *et al*., 2015; Goncalves *et al*., 2014; Reyburn *et al*., 2005; Gupta *et al*., 1999).

As children grow or develop towards the adolescent age, they develop mild disease and thereafter, they progressively become refractory to symptomatic infections. In essence, adults constantly encounter and harbour low level parasites but are asymptomatic, altogether suggesting that complete protection from febrile malaria as observed with other pathogens is remarkably less efficient and that sterilising protection is an impossible feat to achieve in natural populations (reviewed in Langhorne *et al*., 2008; Amanna *et al*., 2007; Hviid, 2005). First time pregnant women living in malaria-endemic regions, however, do not enjoy the protected immunity

acquired from prior and persistent exposure as the presence of a placenta makes them susceptible to certain malaria parasite subsets that preferentially sequester in the placenta leading to severe consequences for both the mother and the foetus. Immunity is, however, built progressively to severe disease with increasing parity (reviewed in Hviid, 2010).

The importance of naturally-acquired immunity from *P. falciparum* malaria has long been recognised. It is believed that antibodies (IgG) are vital components in this response and are directed towards the blood stage parasites where clinical symptoms are observed (reviewed in Fowkes *et al*., 2016; Hviid, 2005; Sabchareon *et al*., 1991; McGregor *et al*., 1963). Significantly, the transfer of purified total IgG from immune Gambian adults to children very ill from malaria rapidly improved the clinical outcome of infection (Cohen *et al*., 1961). The general notion is that non-sterile development of immunity occurs slowly and also fleeting and that ongoing exposure to low-level parasites is an essential requirement to sustain or reinforce immunity, complicating efforts to determine the precise correlates of protection. The slow rate has been attributed to the extensive genetic and antigenic diversity of the parasite (reviewed in Takala *et al*., 2009; Scherf *et al*., 2008) effectively avoiding an immune attack, always a step ahead of the immune system. Thus, many years of frequent exposure to different isolates are required in order to build up a broad spectrum of protective antibodies. Recent accumulating data show that the remarkably slow development is orchestrated by the parasite where it sabotages B cell function, resulting in the generation of the so-called atypical memory B cells that produce ineffective and transient antibodies (Muellenbeck *et al*., 2013; Portugal *et al*., 2013; Weiss *et al*., 2011). Despite these findings, other studies demonstrate that long lasting protective antibody responses which had no bearing on exposure were detectable even

up to 40 years after infection (reviewed in Fowkes *et al*., 2016). Clearly, more research is needed to resolve the issue of the longevity of protective antibody and memory B cell responses as they remain critical in the quest for a potent malaria vaccine.

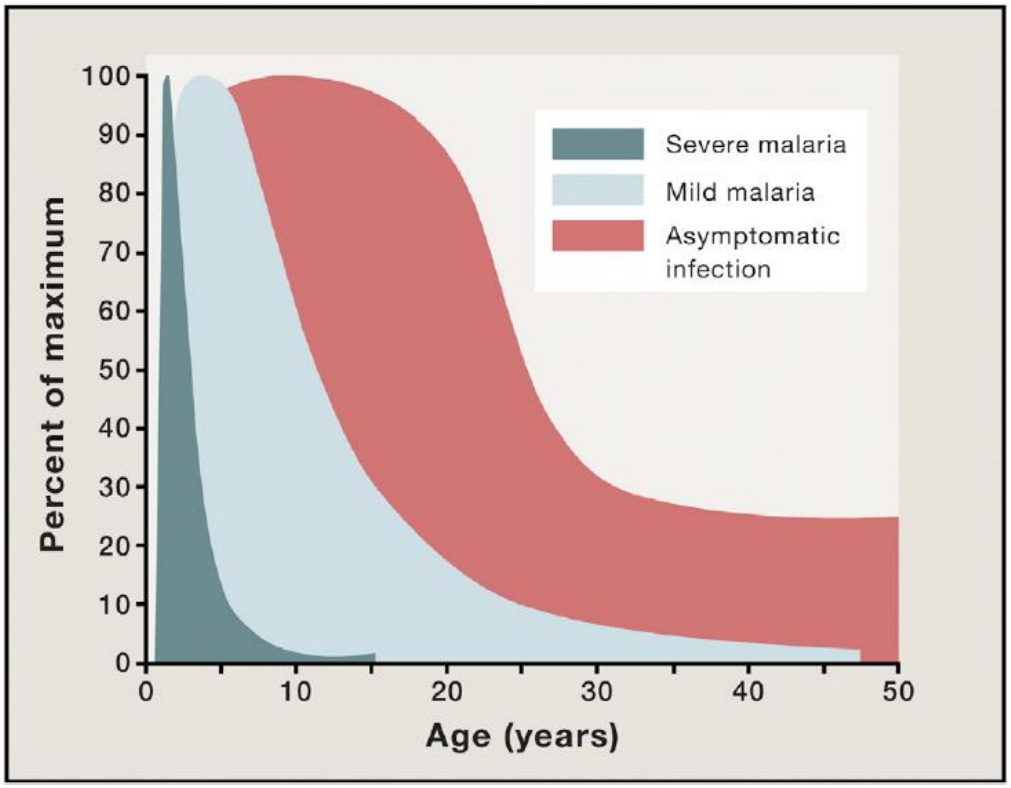


Figure 2.3:The link between age and malaria severity. Acquisition of immunity to the different clinical forms of malaria with increasing age in an area with transmission of *P. falciparum.* (Adapted from Cowman *et al*., 2016).

2.6 Variant surface antigens (VSA) and PfEMP1

Several hours after merozoite invasion of erythrocytes, parasite variable antigens are exported and localised on the surface of infected IEs, presenting the possibility of immune recognition and subsequent attack. These variant surface antigens (VSAs) are encoded by large multigene families concentrated at the telomeres and subtelomeres

and they include repetitive interspersed family (RIFIN) proteins and subtelomeric variable open reading frame (STEVOR) proteins, both of which account for the largest number of genes (~190 genes), *P. falciparum* erythrocyte membrane protein 1 (PfEMP1, 60 genes), surface-associated interspersed gene family (10 genes) proteins and *P. falciparum* Maurer's cleft two transmembrane (12 genes) proteins (reviewed in Chan *et al*., 2014). These VSAs have been implicated in several mechanisms or strategies relating to immune evasion since they undergo antigenic variation, thus allowing parasites to cause persistent infections. Considerable interest has been generated with PfEMP1 presumably resulting from its uniqueness to *P. falciparum*, the species that causes the severe manifestations of malaria, hence it has been the most intensely investigated. However, recent emerging data seem to suggest that the other VSA, particularly RIFIN may have important roles in disease pathology than previously known (Goel *et al*., 2015; Chan *et al*., 2014).

Radioactive labelling of a matured IE with ^{125}I provided proof of the surface location of PfEMP1 on IE and not on the surface of uninfected erythrocytes (Leech *et al*., 1984b). The *var* encoded PfEMP1 is a large, variable molecular weight protein of about 200-350 kDa, sensitive to trypsin treatment which rapidly removes the protein from the surface of IEs (Howard *et al*., 1988; Leech *et al*., 1984b) and it is soluble in sodium dodecyl sulphate implicating its association with the cytoskeleton (Aley *et al*., 1984). Furthermore, PfEMP1 is known to be highly immunogenic with a wide array of antigenic epitopes. Notably, immunoprecipitation experiments demonstrated that sera collected from monkeys that had been infected with a homologous strain agglutinated IEs of the specific strain only and these strain-specific sera were able to interfere with cytoadhesion of the homologous IEs (Howard *et al*., 1988). Expression of PfEMP1 allows infected parasites to be sequestered in various tissues such as the

brain by cytoadhering to diverse host endothelial receptors. It is also involved in rosetting through its interaction with uninfected erythrocytes (Carlson *et al*., 1990) and autoagglutination which results from the platelet-mediated clumping of infected IEs (Pain *et al*., 2001; Roberts *et al*., 2000) [Figure 2.4], thus allowing these parasites to occlude blood vessels and cause local inflammation linked with severity of disease (reviewed in Rowe *et al*., 2009). Cytoadhesion also serves as a strategy that allows the parasite to leave the peripheral circulation and hence avoid spleen mediated killing. More importantly, the ability to express different forms of PfEMP1 provides a smart mechanism to avoid detection by antibodies elicited towards the previous PfEMP1 variant, contributing to the pathogenic nature of *P. falciparum* (Baruch *et al*., 1995).

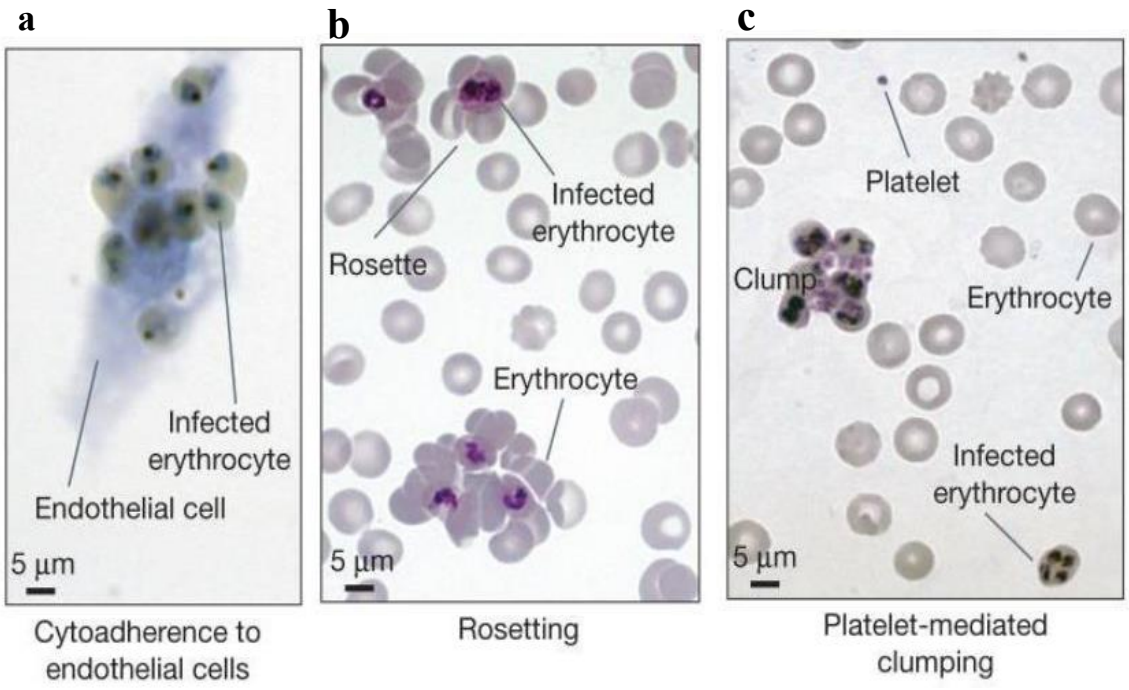


Figure 2.4: Cytoadhesion phenotypes in *P. falciparum*-associated SM pathogenesis. a) Cytoadherence results from the binding of IEs to host endothelial cells. **b)** Rosetting occurs when IEs bind to uninfected erythrocytes. **c)** Platelet-mediated clumping is the process where IEs agglutinate and it is mediated by platelets. Images are observed under a light microscope after Gimesa staining (Adapted from Rowe *et al*., 2009).

2.6.1 *Var* gene architecture

The first complete view and considerable insight into *var* gene architecture and chromosomal organisation was obtained from genomic sequences of the 3D7 reference isolate (Gardner *et al*., 2002). Subsequently, comparative analysis of *var* genes from the IT4 and HB3 genomes with the 3D7 genome revealed a similar number of approximately 60 protein-coding genes per haploid genome (Kraemer *et al*., 2007; Gardner *et al*., 2002). *Var* genes are present on nearly all the parasite chromosome except for chromosome 14, which contains a pseudogene, and they are followed by other multigene families such as RIFIN and STEVOR. Approximately 65 % of the *var* genes are concentrated at the subtelomeric chromosomal regions, reflecting their importance in the survival of the parasite in terms of genetic diversity and variant expression since high recombination events occur at these subtelomeric sites (Gardner *et al*., 2002). These subtelomeric *var* genes are found adjacent to the non-coding telomere repeat elements (1-6) with a considerable majority existing in a tail-to-tail orientation with one or more members of the RIF gene family in between them, while the rest assume or adopt a head-to-head or head-to-tail configuration (reviewed in Scherf *et al*., 2008). The remaining 35 % of *var* genes are located or resident in central clusters on chromosomes 4, 7, 8 and 12 and are frequently found in tandem arrays in a head-to-tail orientation (Gardner *et al*., 2002). With reference to upstream promoter sequences which are strongly linked to specific chromosomal location and direction of transcription, almost all *var* genes fall into four main groups namely: UpsA, UpsB, UpsC and UpsE (Figure 2.5). In addition, there are some subsets falling into intermediates [B/A, B/C] (Kraemer *et al*., 2003; Kraemer and Smith, 2003; Lavstsen *et al*., 2003). Based on the 3D7 parasite genome, there are 10 group A *var* genes (10 genes), all of which are located in the subtelomeric sites and

transcribed toward the telomere (Figure 2.5) in the opposite direction of group B *var* genes (22 genes), which are situated near the telomere and transcribed towards the centromere. Group C *var* genes (13 genes) can be found near the centre of the chromosome. The intermediate groups B/A (4 genes) and B/C (10 genes) have Ups B-like promoters preceding the coding regions of group A or C respectively and are mapped to either subtelomeric or central chromosomal locations. Group A *var* genes are more conserved and larger in size when compared with Groups B and C (reviewed in Hviid and Jensen, 2015; Petter and Duffy, 2015; Lavstsen *et al*., 2003). UpsE consists of a single *var* gene coding for VAR2CSA which is involved in binding to receptors on the placenta during pregnancy-associated malaria (Salanti *et al*., 2003). They are also subtelomeric *var* genes but are transcribed in the opposite orientation to UpsB *var* genes (Rask *et al*., 2010; Kraemer and Smith, 2003; Lavstsen *et al*., 2003; Gardner *et al*., 2002). Overall, *var* gene organisation appears to be very much conserved between parasites of genetically distinct backgrounds (Kraemer *et al*., 2007), and recombination tends to occur between *var* genes from the same 5' flanking sequence and gene orientation, hence maintaining this genetic diversity (Bull *et al*., 2008; Gardner *et al*., 2002).

Var genes share a common architecture encoded in two exon structures and are connected together by an intron. The large hypervariable exon 1 (4-10 kb) consists of a short N-terminal segment (NTS, 75-100 amino acid), multiple Duffy-binding like domains, (DBL, 100-250 amino acid), a cysteine-rich interdomain (CIDR, 70-200 amino acid) and a transmembrane region. Exon II, with a size of about 1.0-1.5 kb, codes for a more conserved intracellular region of the acidic terminal segment which interacts with proteins on the erythrocyte cytoskeleton as well as parasite proteins (Gardner *et al*., 2002; Kyes *et al*., 2001). The intron joining the two exons has a high

adenine and thymine content, a conserved nucleotide sequence and a size of about 0.8-1.2 kb. The DBL and CIDR are highly variable extracellular region with multiple distinct domain structures which have the propensity to bind to several red blood cell adhesion receptors. These adhesion domains have been classified according to conserved residues into various groups that signify distinct binding specificities and phenotypes. DBL domains are categorised into seven types namely α, β, γ, δ, ε, ζ and x. While CIDR domains are grouped into four distinct classes α, β, δ and γ (Rask *et al*., 2010; Smith *et al*., 2000). The DBLα domain is the most abundant and is present in nearly all *var* genes immediately after the N-terminal segment and also harbours most of the conserved *var* sequences found in exon 1 (Kraemer *et al*., 2007; Kyes *et al*., 2007). Although studies have shown that extreme diversity is found within and between *var* genes of *P. falciparum* isolates (Chen *et al*., 2011; Barry *et al*., 2007; Trimnell *et al*., 2006; Bull *et al*., 2005; Fowler *et al*., 2002), the arrangement order of *var* domains is not random. Thus, a conserved head structure has been defined or mapped out and that is composed of NTS-DBLα-CIDR1 domains for most *var* genes except for VAR2CSA and type 3 *var* genes (Kraemer and Smith, 2006; Kyes *et al*., 2001). *Var* genes can also be classified based on the domain cassette (DC) system which consists of combinations of specific DBL and CIDR domain structures occurring in tandem in a PfEMP1 protein (Figure 2.5), thus providing some measure for defining associations with disease phenotypes. Indeed, there is evidence showing that the domain cassette classification of *var* genes has functional relevance with respect to malaria pathogenesis (Warimwe *et al*., 2012).

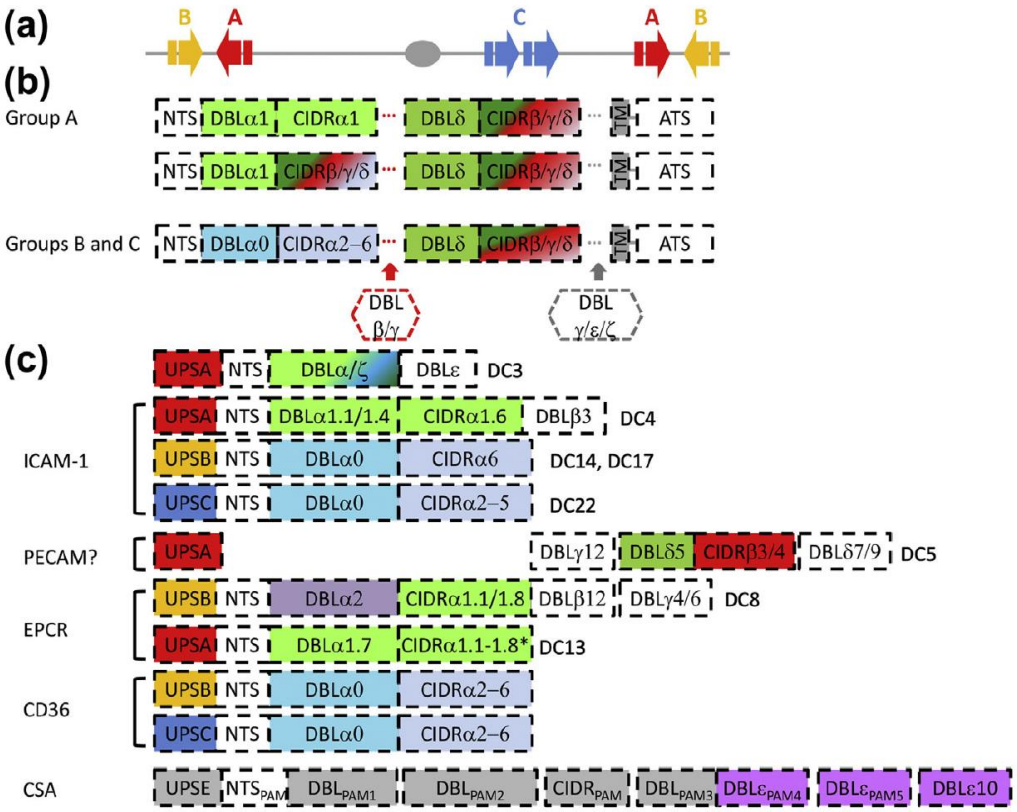


Figure 2.5: *Plasmodium falciparum var* genes chromosomal organisation and PfEMP1 domain structure. **(a)** Group A and B *var* genes occupy subtelomeric chromosomal sites and are transcribed in opposite directions. Group C *var* genes are located in chromosomal central sites. **(b)** PfEMP1 proteins are made up of a conserved non-random head structure that is composed of NTS-DBLα-CIDR1 domains. **(c)** *P. falciparum* genomes encode tandem DBL CIDR domains (domain cassettes; DC). DCs are linked to specific binding phenotypes. For instance, DC4 is involved in ICAM-1 binding. TM, transmembrane; ATS, acidic terminal sequence; UPS, upstream promoter sequence (Adapted from Hviid and Jensen, 2015).

2.6.2 *Var* gene transcription and regulation

Each *var* gene possesses two distinct promoters, one upstream of exon 1 that mediates the expression of a functional messenger ribonucleic acid (mRNA) and is active in the early developmental stages, from 12 to 18 h after invasion. The second is a bidirectional promoter found within the intron and it is responsible for the transcription of non-coding (sterile) RNA mainly by the late-stage parasites and is also

believed to regulate gene expression (Epp *et al*., 2009; Frank *et al*., 2006; Voss *et al*., 2006; Calderwood *et al*., 2003; Deitsch *et al*., 2001b). It is widely accepted that *var* genes are transcribed in the so-called mutually exclusive manner, which allows only one member of the multigene family to be expressed on the surface of an IE while keeping the rest of the members in a state of transcriptional dormancy (Chen *et al*., 1998; Scherf *et al*., 1998). However, one study has demonstrated the capability of dual PfEMP1 transcripts to be expressed on the surface of a single IE (Joergensen *et al*., 2010). *Var* gene transcription or synthesis begins relatively early at the ring stage soon after merozoite invasion of erythrocytes, peaks around 18 h post invasion and further declines to low levels within 20-24 h (Dahlback *et al*., 2007; Kyes *et al*., 2000). Although numerous *var* transcripts have been reportedly identified in the ring stages of malaria parasites, the consensus is that only a single and dominant locus is translated into a functional PfEMP1 protein in matured trophozoites, transported and deposited on the surface of IEs (Chen *et al*., 1998; Scherf *et al*., 1998).

Transport of PfEMP1 through several membranes or compartments occurs via complex and poorly understood mechanisms that involve conserved sequences in the N-terminal, the transmembrane and cytoplasmic domains of PfEMP1 and other partner proteins (reviewed in Przyborski *et al*., 2016; Hviid and Jensen, 2015; Melcher *et al*., 2010). Significantly, PfEMP1 is transported across the parasitophorous vacuole membrane supported by a *Plasmodium* export element (Marti *et al*., 2004), while the parasitophorous vacuole membrane resident *Plasmodium* translocon complex is responsible for translocation into the cytoplasm of the host cell. Trafficking of PfEMP1 is halted around 30-36 h as they are no longer observed on the surface of IEs. The regulation of *var* gene expression is stringent, highly organised and occurs at the transcriptional level in the absence of DNA rearrangements (reviewed in Ralph and

Scherf, 2005; Scherf *et al*., 1998). Regulation of *var* genes at various stages (silent, poised or active) is believed to be orchestrated by tightly interconnected epigenetic-dependent mechanisms owing to the availability of relatively few eukaryotic transcription factors in the *Plasmodium* genome (Figure 2.6). These multilayered epigenetic mechanisms include reversible histone alterations, promoter–intron interaction and sub-nuclear localisation processes (reviewed in Hviid and Jensen, 2015; Guizetti and Scherf, 2013). Deciphering the mechanisms defining *var* regulation may lead to the identification of factors crucially involved in immune evasion and that would provide drug target opportunities.

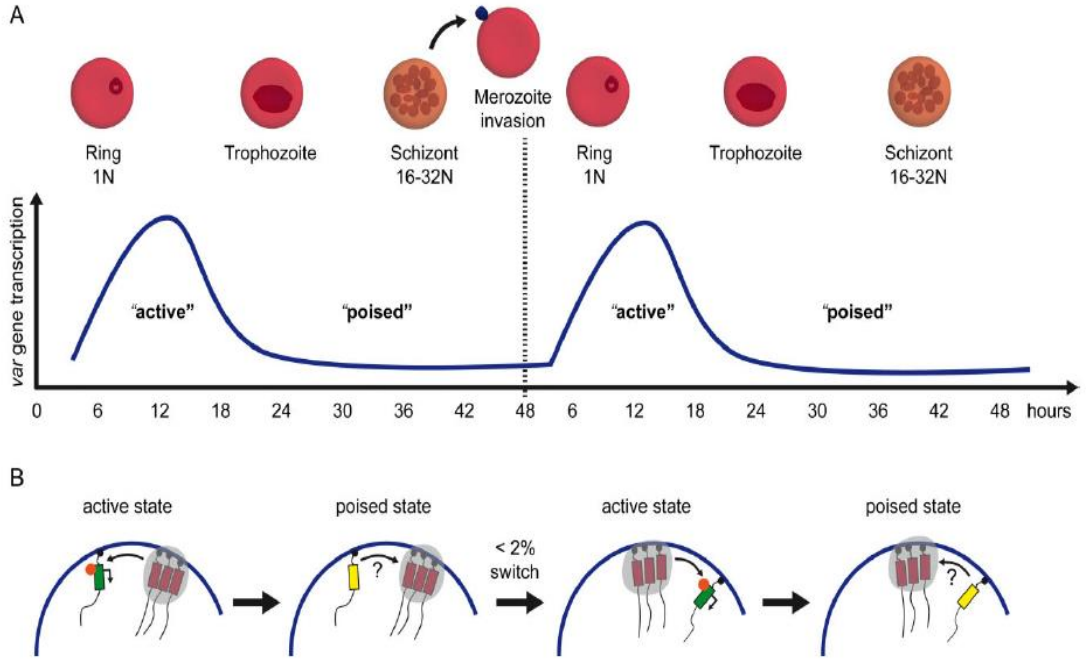


Figure 2.6: *Var* gene regulation in *P. falciparum*. (A).Transcription of a single *var* gene begins at the ring stage following the invasion of erythrocytes by merozoites. Transcription of *var* gene is halted in matured trophozoites and schizonts but remains in a poised state, prepared for activation in the next cycle of erythrocyte invasion. **(B)**. All *var* genes irrespective of their chromosomal position occur in clusters at the periphery of the nucleus (blue). Meanwhile, silent *var* genes group together at perinuclear repressive sites (gray) while the single active *var* gene (green) is found at a dedicated site surrounded by components of euchromatin that will permit its transcription. The mechanism controlling the shift from an active to a poised state is not well elucidated (Adapted from Guizetti and Scherf, 2013).

The nuclear arrangement of *var* genes around the periphery appears to be a fundamental feature of all silenced *var* genes and that occurs regardless of their chromosomal location (Scherf *et al*., 2008). These transcriptionally silent *var* genes are generally found clustered (Figure 2.6) and packaged in condensed chromatin states and known components of repressive heterochromatin seem to be necessary for their maintenance (Lopez-Rubio *et al*., 2009; Ralph *et al*., 2005). Although the molecular details of this perinuclear localisation are largely unknown, Zhang *et al* (2011a) showed that the conserved *var* intron contains a short 18 bp motif which is able to recruit an actin-containing complex to the nuclear periphery with silenced *var* genes (Zhang *et al*., 2011a). Reversible histone alterations play pivotal roles in *var* gene silencing (Lopez-Rubio *et al*., 2007; Freitas-Junior *et al*., 2005). The process involves the removal of acetyl groups on histones, propagated by the Nicotinamide Adenine Dinucleotide (NAD+) dependent Silent Information Regulator 2 (SIR2) family of proteins. Notably, chromatin immunoprecipitation experiments specifically showed the colocalisation of PfSIR2A at a subtelomeric *var* promoter with a silenced *var* gene. Moreover, genetic disruption of SIR2A in parasites resulted in a loss of silencing and subsequent activation of subtelomeric UpsA and UpsB *var* genes (Duraisingh *et al*., 2005). The repressing activity of UpsC *var* genes, however, has been linked to SIR2B suggesting the differential regulation of *var* groups (Tonkin *et al*., 2009). Altogether, SIR2 proteins exert their transcriptional silencing activity possibly by placing a conserved heterochromatin Histone 3 lysine 9 trimethylated (H3K9me3) mark in the promoter regions of repressed *var* genes (Chookajorn *et al*., 2007; Lopez-Rubio *et al*., 2007), which recruits *P. falciparum* heterochromatin protein 1 (PfHP1) and that results in heterochromatin formation and decreased accessibility

for transcription (Coleman *et al*., 2012; Perez-Toledo *et al*., 2009; Duraisingh *et al*., 2005).

Gene silencing involving the single intron connecting exons 1 and 2 in the conserved genetic structure of *var* genes has also been implicated. The intron confers the ability to silence via pairing with its respective upstream promoter (Swamy *et al*., 2011; Calderwood *et al*., 2003; Deitsch *et al*., 2001b), and with the aid of RNA pol II and PfSETvs enzymes which have specificity for the Histone 3 lysine 36 trimethylated (H3K36me3), gene silencing marks are introduced into the two exons (Ukaegbu *et al*., 2014; Jiang *et al*., 2013). Conversely, blocking the interaction or incorporating an unpaired promoter into a silent *var* gene cluster leads to its activation (Avraham *et al*., 2012; Dzikowski *et al*., 2006; Deitsch *et al*., 2001b). Epp *et al* (2009) have also provided evidence of the involvement of non-coding RNA in *var* silencing. The *var* intron promoter mediates bidirectional transcription resulting in long transcripts of sense and antisense non-coding RNA (Epp *et al*., 2009). The expression of the *var* antisense non-coding RNA which are associated with chromatin at telomeric sites stimulates the activation of a silent *var* gene (Amit-Avraham *et al*., 2015). An additional layer of regulation apparently exists at least for specific *var* genes. With reference to that, Zhang *et al*. (2014) recently identified PfRNAse II, a chromatin-associated exoribonuclease known to degrade nascent or short RNA to be enriched at silenced *var* genes. PfRNAse II was shown to silence transcription of group A *var* genes and more importantly, depletion of PfRNase II correlated with elevated expression of group A *var* genes, permitting many group A *var* genes to circumvent silencing (Zhang *et al*., 2014).

At any given time, only a single *var* gene is selectively transcribed for the first 16 h of the parasite's life cycle and mostly maintained in successive generations through

mechanisms not well elucidated (Figure 2.6). Activation of the selected *var* gene involves the replacement of repressive histone marks and loss of PfHP1, which now renders it permissive for transcription (Perez-Toledo *et al*., 2009; Lopez-Rubio *et al*., 2007) . Thus, the promoter region of the active *var* gene is now enriched with loosely packed chromatin which is associated with reversible alterations of histone marks, including Histone 3 lysine 4 bi and tri-methylation (H3K4me2/3) and acetylated Histone 3 lysine 9 (H3K9ac) (Lopez-Rubio *et al*., 2007). An additional modification involved in transcriptional activation include the histone variant H2A.Z. Petter *et al* (2011) showed that H2A.Z is concentrated at the transcriptional start site of active *var* genes and mediates elevated transcriptional activities during the ring stages, and are depleted later in matured stages (Petter *et al*., 2011). Further, H2A.Z-mediated *var* gene activation is antagonised by PfSIR2A silencing factor which results in the removal of PfH2A.Z from the *var* promoter. Again *var* gene activation encompasses the dissociation from silent clusters and relocation of the activated *var* gene to a dedicated nuclear compartment that promotes transcription (Lopez-Rubio *et al*., 2009; Voss *et al*., 2006; Ralph *et al*., 2005)

The transcription of the selected *var* gene locus peaks up around 12 to 16 h post-invasion and then is transiently suppressed during matured schizonts stages (Figure 2.6). The transcribed *var* gene, however, tends to be retained in the so-called poised state and subsequently carried over or inherited in the next cycle of erythrocyte invasion unless a *var* switch occurs. Although the mechanism involving this transition from poised to active state is not well understood, data from chromatin immunoprecipitation experiments indicate that the gene activation mark H3K4me2 which is catalysed by the histone methyltransferase, PfSET10 co-localises with the poised *var* gene post ring stages, presumably to retain the euchromatic epigenetic

memory that will ensure reactivation in the successive cell cycle (Volz *et al*., 2012). It seems that a sophisticated system has been put in place to achieve a regulated *var* gene expression. Nevertheless, some questions remain unanswered and that includes the factors that modulate switching between genes and also what informs or controls the choice and activation of a specific *var* gene.

2.6.3 Antigenic variation and *var* switching

As a means of survival in their host as well as to promote lifelong perpetuation, most pathogenic organisms have evolved a wide range of defense strategies and *P. falciparum* is no exception. Antigenic variation, a process which involves the expression of new subpopulations of clonally variant proteins at regular intervals happens to be one crucially important mechanism employed by parasites to prolong the duration of infection. The first inkling of antigenic variation was observed in *P. knowlesi*, and was then subsequently reported in *P. chabaudi* and sometime later in *P. falciparum* (reviewed in Kyes *et al*., 2001). The parasite's ability to avoid successful destruction by the immune system has been credited to its capacity to generate and sustain diversity through the process of antigenic variation. Malaria parasites accomplish such variation through antigenic alternations between different mutually expressed *var* encoded proteins over the period of an infection (Recker *et al*., 2011; Scherf *et al*., 2008). Frequent alterations of antigenic forms of PfEMP1 result in a switch in receptor usage that enables the parasite to smartly avoid immune destruction. Consistent with that, antibodies elicited to a specific antigen earlier on in the infection or to previous exposures will be ineffective against another variant

expressed in case a parasite switch occurs. This process is responsible for the protracted nature of the infection and it is translated into the classical parasitaemia waves and recrudescences often associated with malaria infections (Petter and Duffy, 2015; Scherf *et al*., 2008; Miller *et al*., 1994). Antigenic variation has been blamed for thwarting the efforts to develop an effective vaccine (Ferreira *et al*., 2007).

During the process of transcriptional switching, a new gene must be selected for activation and that coincides with the simultaneous suppression of the previously active gene. This process appears to be highly organised among members of the *var* family to limit unnecessary immune exposure as well as prevent exhaustion of the entire *var* gene reserves (Ukaegbu *et al*., 2015). Understanding the molecular nuances of *var* gene switching has been extremely difficult because information on epigenetic processes that control antigenic variation is still fragmentary and limited. Despite these challenges, studies from long-term *in vitro* culturing of laboratory parasites and human volunteers infected with parasites have provided significant snapshots on the *var* switching network or dynamics.

Var gene switching rates reported from *in vitro falciparum* cultures occur at an approximately 2 % per parasite generation, irrespective of their chromosomal or promoter occupation (Fastman *et al*., 2012; Frank *et al*., 2007; Roberts *et al*., 1992). By contrast, switching experiments replicated in human volunteers inoculated with malaria parasites reported a high rate of about 16 % per generation (Peters *et al*., 2002). It appears that different switching rates are associated with each *var* gene. Thus, switching is not a random event but rather an organised pattern exists in the choice of which genes are expressed in the event of a switch (Horrocks *et al*., 2004). Furthermore, mathematical models have confirmed the non-randomness of *var* switching which results from a compromise between parasite intrinsic switching and

immune-dependent selection (Recker *et al*., 2011). It is not entirely clear whether external factors or signals can influence *var* switching pattern owing to the dearth of data. Notwithstanding, the relatively few studies suggest that *in vitro* stimuli such as starvation, stress, high temperature or lactate levels can modulate rate and pattern of transcriptional switching (Merrick *et al*., 2012; Rosenberg *et al*., 2009). There is also evidence credited to the immune system in *var* gene alterations. Several investigations have shown that group A *var* genes are more commonly expressed in patients but subsequently change in a random manner to express groups B and C *var* genes when cultured *in vitro*, implicating the role of host factors or immune pressure (Bachmann *et al*., 2011; Zhang *et al*., 2011b; Blomqvist *et al*., 2010; Warimwe *et al*., 2009).

2.7 Endothelial receptors involved in cytoadhesion

A very wide range of host endothelial molecules are known to interact with the extracellular domains of PfEMP1 and currently, about 21 distinct receptors have been reported, reflecting the high variability in PfEMP1 binding behaviour (Esser *et al*., 2014). ICAM-1, CD36 and CSA (Berendt *et al*., 1989; Oquendo *et al*., 1989; Rogerson *et al*., 1995) are among the most extensively characterised and have been shown to be the most widely utilised by *falciparum* malaria parasites. Some receptors are preferentially enriched in some tissues and organs and that has an influence on the type of PfEMP1 expressed to specifically interact with these receptors, leading to the so-called organ-specific syndrome. A classic example is observed in PAM where IE binding to CSA on placental syncytiotrophoblasts has been associated with specific PfEMP1 variants (reviewed in Fried and Duffy, 2015; Salanti *et al*., 2004). In CM, it is believed that specific variants of PfEMP1 bind receptors that are highly expressed

in brain endothelial cells (reviewed in Wassmer and Grau, 2017), although not always consistent. Recent data suggest that individual cytoadhesion domains (domain cassettes) within one PfEMP1 molecule found on the surface of IEs can bind to specific host receptors (reviewed in Hviid and Jensen, 2015). Hence, a complex binding process probably exists where one PfEMP1 is capable of engaging multiple host receptors and interaction with these receptors could result in a synergistic or additive impact or effect between cytoadhesive molecules and this may correlate with disease severity (Adams *et al*., 2014; Aird *et al*., 2014; Yipp *et al*., 2007; Heddini *et al*., 2001).

2.7.1 Intercellular adhesion molecule-1

ICAM-1 is a transmembrane glycoprotein containing five extracellular Ig-like domains and a short cytoplasmic tail (Brown *et al*., 2013; Chakravorty and Craig, 2005; van de Stolpe and van der Saag, 1996). Depending on the level of post-translational glycosylation and cell type, ICAM-1 has a variable molecular weight ranging from 90–115 kDa. It is widely distributed at low levels on several cells including immune cells and endothelial cells. Its expression is significantly increased particularly in brain endothelial cells in response to many proinflammatory cytokines including tumour necrosis factor α (TNF-α) and interleukin-1β (IL-1β) (Amodu *et al*., 2005; Chakravorty and Craig, 2005; Berendt *et al*., 1992). ICAM-1 is well known for stabilising cell–cell interactions and transmigration of leucocytes to sites of inflammation by binding to lymphocyte function-associated antigen (LFA)-1 and macrophage 1 antigen (Mac 1) (van de Stolpe and van der Saag, 1996; Marlin and Springer, 1987). Nevertheless, several ligands have been identified including plasma

fibrinogen, human rhinoviruses and *Plasmodium*-IEs (van de Stolpe and van der Saag, 1996; Languino *et al*., 1993; Berendt *et al*., 1989; Staunton *et al*., 1989). In particular, IEs bind to the N-terminal Ig-like domain of ICAM-1, which is distinct but also overlaps with the LFA-1 binding region (Tse *et al*., 2004; Berendt *et al*., 1992; Ockenhouse *et al*., 1992).

Previous studies from humans and mice have provided strong arguments for the critical involvement of ICAM-1 in SM pathology (Armah *et al*., 2005; Hearn *et al*., 2000). Notably, histological studies by Turner *et al*., (1994) reported increased localisation of IEs and pronounced ICAM-1 expression in brain sections from patients who died from CM. Binding assays reported the adhesion of IEs to ICAM-1 to be significantly higher in CM or other forms of SM in general than in controls, although a direct link to severe disease had been sometimes inconsistent (Turner *et al*., 2013; Ochola *et al*., 2011; Rogerson *et al*., 1999; Newbold *et al*., 1997; Ockenhouse *et al*., 1991). Also, an identified non-synonymous polymorphism in ICAM-1 (ICAM-1^{kilifi}) with high frequency in malaria endemic populations that had differential binding capacity to IEs under flow conditions would have been thought to be protective against severe disease; however, that was not the case as data gathered so far have been conflicting (Fry *et al*., 2008; Jenkins *et al*., 2005; Adams *et al*., 2000; Craig *et al*., 2000; Kun *et al*., 1999; Bellamy *et al*., 1998; Fernandez-Reyes *et al*., 1997). Also, *in vitro* selection of IEs adhering to human brain endothelial cells highly expressed DC 8 and 13 PfEMP1 (Avril *et al*., 2012; Claessens *et al*., 2012) that failed to bind ICAM-1.

On the parasite side, available data suggest that ICAM-1 binding has evolved into two binding lineages. Group A DBLβ associated with severe disease, with a particular interest in the discovery of DC4, a group A PfEMP1 that binds to ICAM-1 (Bengtsson

et al., 2013b; Oleinikov *et al*., 2009). DC4 was discovered through orthologic search of the 3D7 *var* gene PFD1235w in parasite isolates from Ghanaians with malaria (Bengtsson *et al*., 2013b) and it comprises three domains (DBLα1.1/1.4-CIDRα1.6-DBLβ3). IEs selected *in vitro* to express DC4 mediated ICAM-1 binding but not binding to CD36 and experiments using chimeric protein constructs revealed the ICAM-1 interaction site in DBLβ3, a region located in the C-terminal portion (Bengtsson *et al*., 2013b). DC4 DBLβ3 antibodies are acquired naturally in early age and they have been shown to be cross-reactive and cross-inhibitory of DC4 containing ICAM-1 binders across different *falciparum* genomes (Bengtsson *et al*., 2013b). Furthermore, Lennartz *et al* (2015) characterised a monoclonal antibody 24E9 that targeted conserved epitopes of DC4 DBLβ3 parasites from different parasite genomes by blocking or interrupting ICAM-1 interaction site on DBLβ domain. Thus, the accumulated data provided the feasibility of considering these DBLβ proteins in the design of a PfEMP1 based vaccine.

The other lineage involves DBLβ PfEMP1 in groups B and C and this forms a group of ICAM-1 binders that have the capacity to synergise with CD36 to enhance firm endothelial adhesion, a binding phenotype linked with UM (Janes *et al*., 2011; Ochola *et al*., 2011; Howell *et al*., 2008; Yipp *et al*., 2000; McCormick *et al*., 1997; Cooke *et al*., 1994). Altogether, these data suggest that ICAM-1 binding alone may not be sufficient in causing CM. Indeed, we recently provided more evidence using bioinformatics and structural biology in identifying a conserved sequence (motif) in PfEMP1 that allowed us to predict ICAM-1 binding (chapter three). Comparative analysis of many parasite genomes revealed that these PfEMP1 that mediated ICAM-1 binding possessed also an EPCR-binding CIDRα1 domain suggesting that PfEMP1 with this motif could synergistically engage both receptors (Lennartz *et al*., 2017).

Although, it has previously been reported in only one study that some groups of PfEMP1 contain domains that can interact both with EPCR via CIDRα1 and ICAM-1 via DBLβ domain (Avril *et al*., 2016), we showed in more detail the dual and simultaneous binding ability of these PfEMP1 constructs containing both ICAM-1 and EPCR binding domains with high affinity in surface plasmon resonance experiments (Lennartz *et al*., 2017).

Furthermore, IEs expressing PfEMP1 predicted to bind to both receptors were selected *in vitro* and binding assays under flow conditions provided evidence for increased adhesion in the presence of both receptors in comparison with either receptor alone (Lennartz *et al*., 2017; Chapter three). Quantitative real-time PCR (qPCR) data showed significantly high transcription of motif-containing ICAM-1 binders in CM compared with SA (SA) and other forms of SM (Lennartz *et al*., 2017; Chapter three). Thus, this study provides evidence for the first time of the ability to predict group A ICAM-1 binders based on sequence information and also showed that parasites expressing this PfEMP1 motif interacted with both ICAM-1 and EPCR and had a link with CM development (Lennartz *et al*., 2017; Chapter three).

Membrane-associated ICAM-1 is believed to be cleaved from cellular surfaces into circulation since soluble forms consist of only the extracellular portion of ICAM-1. Soluble ICAM-1 (sICAM-1) has been quantified in plasma samples and the physiological concentration falls between 200-300 ng/mL. However, these levels have been found to be markedly high in several disease manifestations including malaria (Chakravorty and Craig, 2005; Gearing and Newman, 1993). Several studies have examined the levels of sICAM-1 in *falciparum* infected patients and the consensus is that the levels are higher in SM compared with UM or controls but the levels do not significantly discriminate between the different types of SM syndromes (Cserti-

Gazdewich *et al*., 2010; Tchinda *et al*., 2007; McGuire *et al*., 1996; Jakobsen *et al*., 1994; Hviid *et al*., 1993) [Chapter five]. High levels of sICAM-1 in plasma of malaria patients may signify the combined efforts of excessive inflammatory response, increased activation of endothelial cells and decreased removal of sICAM-1 from the circulatory system (Cserti-Gazdewich *et al*., 2010).

2.7.2 Endothelial Protein C Receptor

The human endothelial protein C receptor, EPCR, also known as activated protein C (APC) receptor and encoded by the *proc* gene is a 46 kDa type 1 transmembrane protein that is structurally related to the major histocompatibility class 1/CD1 family of proteins (reviewed in Rao *et al*., 2014). EPCR expression is found on diverse cells including immune cells and endothelial cells, with particularly low expression on the brain endothelium or microvascular cells (Laszik *et al*., 1997). The endothelial cell-bound EPCR functions as a protein C receptor which results in protein C activation via thrombin-thrombomodulin complex signalling. APC interacts with Protease-activated receptor 1 (PAR1) which is involved in regulation of inflammation, coagulation and neuroprotective processes (Rao *et al*., 2014; Griffin *et al*., 2012; Stearns-Kurosawa *et al*., 1996; Fukudome and Esmon, 1994). EPCR is the most recently discovered receptor implicated in SM pathology and evidence to support this role was provided by two independent studies. IEs selected to bind brain endothelial cells expressed PfEMP1 encoding DC8 and DC13 (Avril *et al*., 2012; Claessens *et al*., 2012) and were observed to adhere to EPCR (Turner *et al*., 2013).

Moxon *et al* (2013), while examining samples of children who died from CM showed the shedding of the normally low levels of membrane-associated EPCR coupled with

fibrin deposits localised with sequestered IEs in brain microvascular sections. More studies have clearly shown that parasite isolates from adults and children suffering from SM had high transcription levels of DC8 and DC13 (Mkumbaye *et al*., 2017; Bernabeu *et al*., 2016; Almelli *et al*., 2014; Bertin *et al*., 2013; Lavstsen *et al*., 2012) with differential binding characteristics or ability to bind EPCR. EPCR interacts with high affinity with CIDRα1.1 and the α1.4-1.8 domains of PfEMP1 and indeed, Jespersen *et al.,* (2016) analysed almost full-length sequences of PfEMP1 from parasites obtained from paediatric SM cases from Tanzania and found the predominance of EPCR-binding CIDRα1 transcripts. More importantly, antibodies targeting CIDRα1.1 regions have been reported to be highly acquired in residents from malarious countries at an early age, further buttressing the key involvement of EPCR in SM (Turner *et al*., 2015).

Recently, the molecular determinants for PfEMP1 interaction with EPCR were elucidated. Using co-crystals of soluble EPCR sEPCR bound to PfEMP1 CIDRα1 domains, the binding site of PfEMP1 was revealed and that region had a considerable overlap with that of the protein C interaction site on EPCR (Turner *et al*., 2013; Lau *et al*., 2015). Altogether, the data suggest that in the presence of PfEMP1 parasites, the anti-coagulant state of the endothelium via activation of protein C signalling may be competitively disrupted (Turner *et al*., 2013; Lau *et al*., 2015). Indeed, *in vitro* studies demonstrated that PfEMP1 interaction with EPCR attenuated the activation of protein C as well as the downstream signaling of EPCR-dependent PAR1 activation, resulting in PAR1-dependent compromise of the vascular barrier integrity. This provides a plausible mechanism connecting sequestration, coagulation defects and endothelial activation to SM pathogenesis (Gillrie *et al*., 2015; Petersen *et al*., 2015).

A plasma soluble form (sEPCR) which results from metalloproteinase-mediated cleavage of the membrane bound EPCR is also present in healthy individuals at very low concentration (Qu *et al*., 2007; Xu *et al*., 2000; Kurosawa *et al*., 1997). Soluble EPCR competitively binds to protein C with similar affinity (Fukudome *et al*., 1996; Regan *et al*., 1996). The levels of sEPCR in plasma are believed to be influenced by variations in the genetic sequence of EPCR. Significantly, an exon 4 variant (rs867186-G), with a serine-to-glycine substitution at position 219 appears to be associated with increased shedding of the membrane-bound form into circulation (Uitte de *et al*., 2004). Many studies have assessed the levels of sEPCR as well as the rs867186-GG variant in an attempt to link these levels with severity of many infectious diseases such as venous thrombosis (Medina *et al*., 2005; Medina *et al*., 2004). In malaria, the data shown so far are not clear cut presumably because few studies have been carried out on the relatively new receptor. Moussiliou *et al* (2015) reported elevated levels of sEPCR in Beninese children diagnosed with CM compared with controls. However, other studies showed reduced levels of sEPCR in SM compared with UM or community controls (Shabani *et al*., 2016; Hansson *et al*., 2015) and surprisingly higher levels recorded in convalescent samples in one study (Hansson *et al*., 2015).

In this thesis, significantly elevated sEPCR levels were observed in malaria patients in general compared with healthy community controls (Chapter five). With regard to genetic studies, data from one study in Africa found that the rs867186-GG genotype was associated with increased levels of sEPCR and these levels mediated protection from SM (Shabani *et al*., 2016), whereas two other African studies found no link between the rs867186-GG genotype and protection from severe disease (Hansson *et al*., 2015; Schuldt *et al*., 2014). The only study conducted in Asia seems to suggest

that this particular EPCR genetic variant offer protection against SM (Naka *et al*., 2014).

2.7.3 Cluster of Differentiation 36

The class B scavenger receptor member, CD36, is an 88 kDa transmembrane glycoprotein widely expressed on endothelial and epithelial cells, macrophages, monocytes, platelets and adipocytes. It is keenly involved in fatty acid metabolism, innate immunity and angiogenesis (Febbraio *et al*., 2001; Greenwalt *et al*., 1992). Of all receptors recognised to bind to IE, CD36 binding seems to be the most frequently observed in almost all *P. falciparum* isolates spanning the various clinical categories or presentations and is perhaps advantageous to parasite survival (reviewed in Rowe *et al*., 2009; Serghides *et al*., 2003; Oquendo *et al*., 1989). Besides endothelial sequestration, IE binding to CD36 has been linked to both macrophage-mediated phagocytosis necessary to control parasite density (McGilvray *et al*., 2000) and also regulating dendritic cell function, in order to hamper immune response (Urban *et al*., 1999). Despite being one of the most extensively studied, its role in malaria pathogenesis remains highly unresolved. On one hand, studies from Africa explicitly show that CD36 binding in parasites obtained from SM is similar to parasites from UM (Rogerson *et al*., 1999; Newbold *et al*., 1997) and moreover results from human population genetics show no association (Fry *et al*., 2009). Reports from Asia on the other hand, are very conflicting since some data report a link to SM (Ockenhouse *et al*., 1991) and that CD36 mutations confer protection against severe disease (Omi *et al*., 2003).

Since there is no clear data showing the direct involvement of CD36 in SM or protective mechanisms against severe disease, it is mostly accepted that they are more relevant in uncomplicated and asymptomatic malaria. Indeed, preferentially high

transcription of groups B and C *var* genes coding for CD36 binding phenotypes have been observed from parasite isolates of non-SM (Lavstsen *et al*., 2012; Kyriacou *et al*., 2006). Binding to CD36 occurs via CIDRα2-6 domains of groups B and C PfEMP1 and often times partnered by ICAM-1 (Ochola *et al*., 2011; Janes *et al*., 2011; Robinson *et al*., 2003; Baruch *et al*., 1997). Moreover, CD36 is hardly detected in the brain microvasculature, as IEs that adhere to HBEC have no affinity for CD36 binding, thus making it impossible for CD36 to be a key culprit in cerebral sequestration (Avril *et al*., 2012; Claessens *et al*., 2012; Turner *et al*., 1994). These data do not, however, offer any explanations on the ubiquitous CD36 binding observed in the vascular beds of many non-vital tissues including the skin, adipose tissue and muscle of individuals suffering from non-SM.

Recently, the key structural requirements that define the widespread binding nature of IEs to CD36 were elucidated. Based on structural and biophysical analyses of CIDR sequences, Hsieh *et al*., (2016) revealed that a critical phenylalanine residue that extends from the surface of CD36 fits into a conserved hydrophobic pocket in PfEMP1. The binding cavity or pocket which is concave shaped and also highly unnoticeable or shielded suggests that it may be a difficult target for antibodies. It appears that the conserved phenylalanine residue is important for CD36 to carry out its biological function in fatty acid metabolism. Hence, it is nearly impossible to disrupt or alter this targeted region, since that may have detrimental or grave consequences on host metabolism (Hsieh *et al*., 2016). Altogether, it shows how smartly the parasite has capitalised on a physiologically relevant host protein by using its polymorphic PfEMP1 protein in order to promote its survival by eluding immune destruction. This complex mechanism to some extent explains the reason behind CD36 being the most frequently encountered or preferred parasite receptor in non-

vital organs. The process may facilitate parasite perpetuation as well as reduce host morbidity and mortality which are often associated with non-SM (Hsieh *et al*., 2016; Cabrera *et al*., 2014).

2.8 PfEMP1 in disease and immunity

It is largely agreed in the literature that the PfEMP1 proteins are crucially important targets of immunity (Bull and Abdi, 2016; Hviid, 2010; Lusingu *et al*., 2006; Ofori *et al*., 2002; Bull *et al*., 1998). Parasite isolates from SM patients with low immunity seem to express a restricted subset of groups A and B/A PfEMP1 and these are more widely recognised by antibodies from older children who have experienced frequent bouts of *P. falciparum* malaria. Conversely, high transcription of PfEMP1 from group C have been observed from parasites isolated from older children with UM and asymptomatic infections (Warimwe *et al*., 2009; Kaestli *et al*., 2006; Kyriacou *et al*., 2006; Rottmann *et al*., 2006; Jensen *et al*., 2004; Nielsen *et al*., 2002; Ofori *et al*., 2002). More importantly, antibodies to recombinant PfEMP1 domains are recognised in a particular manner which is a non-random order, of which group A PfEMP1 variants associated with severe disease are firstly recognised (Cham *et al*., 2010; Cham *et al*., 2009). Thus, it can be inferred from these studies that some particular subsets of PfEMP1 molecules are preferentially transcribed by parasites causing SM. Indeed, recently accumulating data show that DC 4, 5, 8 and 13 which are members of group A or B/A PfEMP1 are linked with SM. Antibodies against these recombinant DCs are acquired early in life and appear to correlate with protection from severe disease (Turner *et al*., 2015; Bengtsson *et al*., 2013b; Bertin *et al*., 2013; Lavstsen *et al*., 2012; Magistrado *et al*., 2007). Together, these highlight the possibility of

developing a PfEMP1 based vaccine aimed at reducing SM associated mortality and morbidity in endemic areas.

2.9 Pathogenesis of CM

It is well recognised that CM pathology is complex and is thought to be related to several factors including parasite cytoadhesion accompanied by a blockage in the cerebral microvasculature, imbalanced inflammatory immune responses and endothelial activation. Cytoadhesion is mediated by variant parasite surface proteins to multiple host proteins, while host cells including leucocytes, endothelial cells and platelets mediate the inflammatory response (Storm and Craig, 2014; Miller *et al*., 2013; Wassmer *et al*., 2011; Berendt *et al*., 1994; Clark and Rockett, 1994). The combined efforts of sequestration and inflammation are thought to drive endothelial activation resulting in vascular leakage, a compromise of brain barrier integrity and brain swelling leading to death (Seydel *et al*., 2015; Grau and Craig, 2012). It is entirely unknown which of the factors initiate the pathological processes. Also, the relative involvement or contribution of each of these events has been impossible to investigate and that has frustrated efforts to develop some form of supportive therapy and vaccine.

2.9.1 The sequestration model

A prominent observation reported from detailed analysis of CM autopsy samples is an increased mass of sequestered IEs in brain cells (Milner, Jr *et al*., 2014; Taylor *et al*., 2004; Silamut *et al*., 1999; MacPherson *et al*., 1985), thus providing a link between

sequestration and CM. Sequestration, however, appears not to be exclusive to CM as it has also been observed in other organs including the heart and the small intestines with no pronounced pathology (Pongponratn *et al*., 2003). Sequestration results from the cytoadhesion of particular PfEMP1 variants including DC4, DC8 and DC13 to host receptors such as ICAM-1 and EPCR (Figure 2.7) on brain endothelial cells and that impedes the flow of blood, causes hypoxia and reduced clearance of toxic lactic acid (Hviid and Jensen, 2015; Dondorp, 2008; van der Heyde *et al*., 2006). Indeed, significantly increased levels of blood lactate concentrations correlate with CM development (Marsh *et al*., 1995). Also, there is a reduction in the deformability of IEs and uninfected erythrocytes leading to capillary plugging and that process has been linked to poor disease outcome (Dondorp *et al*., 2000). A number of important observations cannot be accounted for by the sequestration model. Information on the molecular details of CM pathogenesis is usually obtained from studies in *P. berghei*. Data from these studies show that cerebral IE sequestration is not associated with CM development but rather inflammatory processes are the key players (reviewed in Craig *et al*., 2012; Cabrales *et al*., 2010; de Souza *et al*., 2010). The brain-associated impairments reported in CM survivours are low compared to other brain-associated pathologies that can result in brain anoxia (reviewed in van der Heyde *et al*., 2006; Clark and Cowden, 2003).

2.9.2 The inflammation model

During the asexual blood stage of malaria, parasites or their toxic waste products (haemozoin and glycophospatidyl inositol) which are liberated during the lysis of erythrocytes provoke cells of the immune system as well as endothelial cells to secrete

both pro- and anti-inflammatory cytokines such as IL-1, IL-6, IL-10, TNF-α and neuro-active mediators including nitric oxide (Schofield and Grau, 2005; Gazzinelli and Denkers, 2006). The inflammatory cascade involving activated immune cells and secreted cytokines, in turn, mobilise CD4+ and CD8+ T cells, all in an attempt to contain the infection. Notably, most of the malaria symptoms such as fever, nausea and headaches are the consequences of the inflammatory response in its attempt to control parasite replication and ultimately resolve the infection (Hunt *et al*., 2006; Schofield and Mueller, 2006; Urban *et al*., 2005; Stevenson and Riley, 2004). However, uneven ratios between pro- and anti-inflammatory responses to malaria infection resulting from improper regulation could be detrimental to the host and pathology of severe disease has been consistently associated with excessive systemic and local inflammation (Lyke *et al*., 2004; Dodoo *et al*., 2002; Day *et al*., 1999; Kwiatkowski *et al*., 1990; Grau *et al*., 1989).

Accumulating evidence on the inflammatory mechanisms contributing to the induction of severe disease has been extrapolated from the murine *Plasmodium berghei* ANKA model and clinical studies in humans (reviewed in Hansen, 2012; Higgins *et al*., 2011; de Souza and Riley, 2002). Significantly, studies from knockout mice and inhibitory monoclonal antibodies demonstrate that the inflammatory response is central to the development of disease (reviewed in Schofield and Grau, 2005). In addition, elevated circulating levels of TNF-α and other cytokines such as high mobility group box 1 protein (HMGB1) present in the serum of severe patients significantly correlated with CM, were observed (Alleva *et al*., 2005; Lyke *et al*., 2004; Kwiatkowski *et al*., 1990). Observations of brain sections of CM autopsies revealed deposition of TNF-α and interferon γ [IFNγ] (Udomsangpetch *et al*., 1997). These cytokines are thought to contribute to disease severity by increasing the

expression level of cytoadhesion molecules such as ICAM-1, hence enhancing the binding of IEs to endothelial cells (Wassmer *et al*., 2005; Ho and White, 1999; Ockenhouse *et al*., 1992). The inflammation model has failed to explain some aspects of CM development; the elevated concentrations of mediators such as TNF-α recorded in patients with uncomplicated *vivax* infections and also the failure of anti-inflammatory agents to offer beneficial effects on disease severity but rather worsen the clinical course in some situations (Anstey *et al*., 2009; van Hensbroek *et al*., 1996). In summary, the data suggest that inflammatory cytokines may be important but not sufficient for CM development.

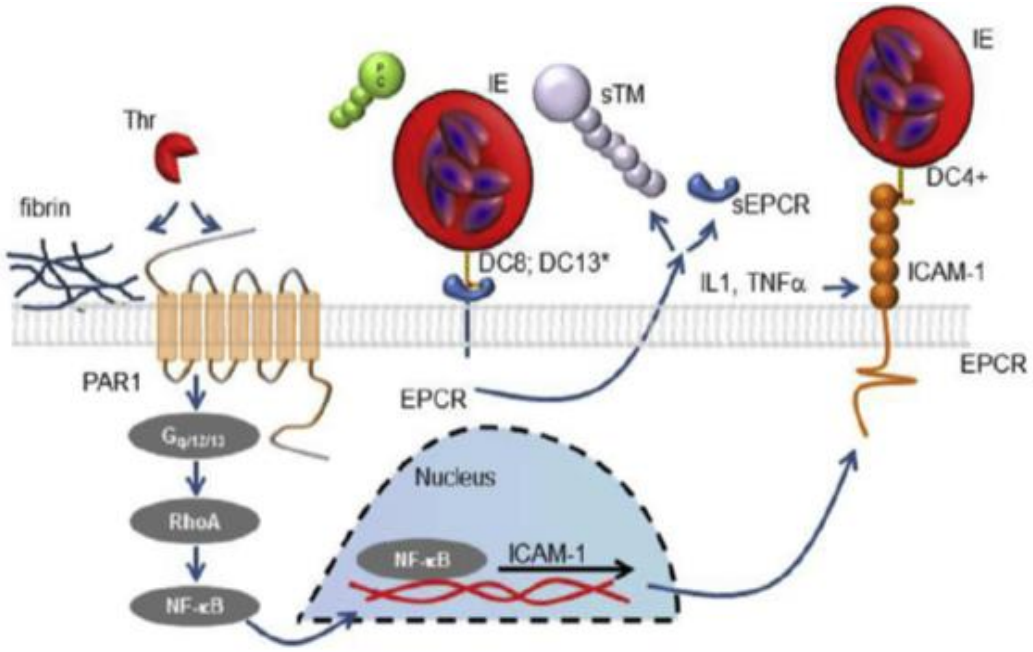


Figure 2.7: A possible mechanism of ICAM-1 and EPCR cooperation in CM pathogenesis. DC8 or DC13-expressing IEs bind to EPCR resulting in stimulation of endothelial cells and release of pro-inflammatory cytokines such as TNF-α which leads to loss of EPCR and thrombomodulin from the surface of endothelial cells with subsequent activation of pathways that promote high expression of ICAM-1 molecules. IEs expressing either DC4 or containing the group A PfEMP1 ICAM-1 motif bind ICAM-1. The initial binding could also be mediated by ICAM-1 binding parasites containing the EPCR CIDR binding domain and that will lead to increased expression of ICAM-1 resulting in increased cytoadhesion, endothelial activation, compromise in barrier activities, brain swelling and death (Adapted from Hviid and Jensen, 2015)

2.9.3 Endothelial activation

The endothelium consists of a monolayer of endothelial cells lining the vascular system and approximately 10^{13} endothelial cells can be found in a human adult. Endothelial cells together with pericytes, basal lamina, astrocytes and perivascular endfeet form critical components of the blood–brain barrier (reviewed in Abbott *et al*., 2006; Medana and Turner, 2006; Galley and Webster, 2004). A healthy endothelium produces nitric oxide which mediates vasodilation, downregulation of endothelial adhesion molecules, anti-inflammatory and antithrombotic processes (Bergmark *et al*., 2012). In malaria, the endothelium provides a platform where IEs interact with the human host with particular importance in CM where IEs are sequestered in cerebral endothelial microvessels. Endothelial activation appears to be mediated by multiple host and parasite factors (reviewed in Cunnington *et al*., 2013b; Miller *et al*., 2013; Grau and Craig, 2012; Kim *et al*., 2011) which intensify the pathological processes of malaria resulting in damage and apoptosis of ECs, vascular leakage, brain oedema and possibly death (reviewed in Seydel *et al*., 2015; Shikani *et al*., 2012). In addition, the repair mechanism of the endothelium is compromised due to significantly low levels of circulating endothelial progenitor cells (Gyan *et al*., 2009). Endothelial activation appears to be a prominent feature in CM pathogenesis (Conroy *et al*., 2010; Medana and Turner, 2006; Turner *et al*., 1998; Turner *et al*., 1994). It promotes high expression of some of its surface adhesion molecules which in turn are shed into the plasma. For instance, ICAM-1 is known to be significantly expressed on brain endothelial cells and is colocalised with sequestered IEs (Turner *et al*., 1994). Thus, endothelial activation upsets the vascular integrity by promoting increased IE sequestration through the overexpression of surface adhesion molecules and also by amplifying the inflammatory signals, forming the so-called positive feedback loop

(Cunnington *et al*., 2013b; Kim *et al*., 2011). Furthermore, activated endothelial cells lead to rapid exocytosis and release of activation markers such as von-Willebrand factor and angiopoietin-2 (Ang-2) from Weibel-Palade bodies into circulation (Matsushita *et al*., 2005), as a result of reduced nitric oxide bioavailability which usually prevents the release of Ang-2 (Hanson *et al*., 2015; Yeo *et al*., 2014). Endothelial activation and the subsequent dysfunction observed in severe *falciparum* malaria has also been reported in severe *P. vivax* malaria (Barber *et al*., 2015; Yeo *et al*., 2010), suggesting the non-exclusivity of this process.

The Angiopoietin (Ang) family of proteins and their Tie-2 receptor are well known for their basic physiological assignment in regulating the endothelial barrier integrity (Conroy *et al*., 2012). Ang-1-mediated Tie-2 signalling is expressed constitutively and maintains the endothelium in a steady state, thus suppressing inflammation and preventing vascular leakage. Ang-2, however, acts as an antagonist to the protective effects of Ang-1 (Fiedler and Augustin, 2006). During endothelial activation resulting from impaired endothelial nitric oxide production, Ang-2 is rapidly released from endothelial cells and binds competitively to Tie-2 by displacing Ang-1. Consequently, Ang-2 signalling disrupts the stability of the endothelium by facilitating the loss of barrier integrity. Specifically, Ang-2 primes endothelial cells to be activated by other inflammatory cytokines including TNF-α and IL-1, thereby destabilising the microvascular integrity and resulting in increased vascular permeability (Bernabeu and Smith, 2017; Hora *et al*., 2016; Shikani *et al*., 2012; Fiedler and Augustin, 2006). A dysfunctional endothelium appears to be an important step in the progression to severe disease including fatal CM and this process is gaining widespread investigation. Notably, previous studies have collectively reported reduced circulating Ang-1 levels and increased Ang-2 levels in severe patients compared to

uncomplicated malaria patients (Conroy *et al*., 2012; Conroy *et al*., 2009; Lovegrove *et al*., 2009). Indeed, plasma Ang-1 and Ang-2 levels have been shown to be powerful predictive clinical biomarkers which are able to differentiate paediatric CM and retinopathy-positive patients and also provide information on the degree of retinal damage (Conroy *et al*., 2012).

2.10 PfEMP1-mediated IgM binding and disease severity

Besides mediating adhesion to endothelial receptors, another striking feature of IEs expressing PfEMP1 is their ability to bind to soluble serum factors including non-immune IgM. Binding of non-immune IgM has been strongly connected to virulent *P. falciparum* phenotypes (Barfod *et al*., 2011; Creasey *et al*., 2003; Rowe *et al*., 2002). Transmission electron microscopy experiments provide initial evidence of fibrillar structures partly comprised of IgM from non-immune individuals linking IEs to uninfected erythrocytes in rosette formation (Scholander *et al*., 1996). Subsequent experiments have extensively described how IgM-mediated rosetting and more importantly anti-IgM antibodies to some extent can interfere with rosette formation (Clough *et al*., 1998; Scholander *et al*., 1996). Further studies demonstrated the occurrence of IgM binding in IEs that adhere to CSA in placental malaria (Creasey *et al*., 2003). Specifically, IEs expressing VAR2CSA, a PfEMP1 variant is responsible for binding to CSA (Salanti *et al*., 2004; Salanti *et al*., 2003). Although these placental IEs did not form rosettes, it appeared that they had dual capacities to bind to CSA and IgM (Barfod *et al*., 2011; Rasti *et al*., 2006; Creasey *et al*., 2003). The Fc portion (Cμ4 domain) of the human pentameric IgM is involved in binding to IEs expressing

PfEMP1. The IgM binding site in PfEMP1 has been mapped to specific DBLζ and DBLε domains (Stevenson *et al*., 2015a; Ghumra *et al*., 2008; Semblat *et al*., 2006). Although the molecular basis, as well as the physiological relevance of IgM binding to PfEMP1, is not fully understood (reviewed in Pleass *et al*., 2016; Czajkowsky *et al*., 2010), in rosetting for instance, it is thought that IgM together with other serum proteins 'cross-bridge' and align multiple PfEMP1 molecules thereby tightening and possibly counteracting their relatively weak interactions with carbohydrate receptors on erythrocytes (Stevenson *et al*., 2015a; Stevenson *et al*., 2015b). By contrast, IgM binding to VAR2CSA seems to conceal important PfEMP1 epitopes targeted for phagocytic destruction, cleverly eluding immune destruction which is beneficial to parasite survival (Barfod *et al*., 2011).

Some PfEMP1 domain subtypes have been repeatedly documented to bind IgM (Stevenson *et al*., 2015a; Ghumra *et al*., 2008; Rasti *et al*., 2006; Semblat *et al*., 2006). Given the clinical relevance of IgM binding, Jeppesen *et al* (2015) set out to investigate the number of IgM binding PfEMP1 in the *P. falciparum* NF54/3D7 parasite genome using the NF54-G6 clonal parasites, which have their *var* genes completely erased without affecting the viability or survival of the parasite. Briefly, *P. falciparum* G6 clonal parasites were transfected with the plasmid pVBH, which bypasses the mechanism that governs mutually exclusive *var* gene expression but drives the expression of blasticidin S deaminase and essentially allows the reset of PfEMP1 expression by deleting the epigenetic memory of *var* gene transcription. Thus, beginning with parasite clones with an almost non-existent v*ar* background and removal of the blasticidin pressure, transcription is induced which is accompanied by the expression of nearly all *var* genes in the parasite genome (Fastman *et al*., 2012; Dzikowski and Deitsch, 2008; Frank *et al*., 2007). These parasites can then be IgM

selected, single sorted based on the ability to bind IgM which can then be further functionally characterised. Taking advantage of this technology, Jeppesen and colleagues (2015) showed that a minimum of five IgM binding PfEMP1 are present in the genome of NF54/3D7 parasite. An important finding from this study was that the functional role of the identified PfEMP1 IgM binders was not limited to mediating rosetting and CSA adhesion, suggesting unknown mechanisms for IgM binding to other PfEMP1 variants. One possible mechanism may be to increase the host receptor binding arsenal involved in sequestration; hence circumvent immune detection and clearance (reviewed in Pleass *et al*., 2016). It appears that IgM binding may be an important phenotype (Jeppesen *et al*., 2015) and its numbers in the parasite genome may have been possibly underestimated. It is therefore pertinent to explore the possibility of discovering and characterising new PfEMP1 variants that bind IgM (Chapter seven) since such information may be relevant in guiding the development of interventions and also narrow the gap in the understanding of these adhesive interactions.

2.11 Blood outgrowth endothelial cells (BOECs)

In order to elucidate the pathogenesis of SM to facilitate the development of interventions, it is very important to have a model that allows the monitoring of malaria right from parasite infection through the various stages to full blown disease manifestation. Human autopsy evaluations, *in vivo* animal models and *in vitro* cells have been the mainstream approaches in studying SM (Milner, Jr. *et al*., 2015; El-Assaad *et al*., 2014; Craig *et al*., 2012; Taylor *et al*., 2004). Although these studies provide invaluable descriptive data, they have obvious drawbacks. Postmortem

analysis evaluates tissues at end point, usually comparing non-fatal cases to fatal cerebral cases. In addition, access to samples is challenging because of ethical reasons, making this approach very unattractive (White, 2011; Medana *et al*., 2001). *In vivo* animal models have great advantages as they permit the monitoring of parasite development from inoculation to its transmission and have offered significant insights especially on the host immune factors. Unfortunately, the currently available models such as the rodent *P. berghei* do not fully replicate the pathophysiological features of CM as *P. falciparum* is only able to infect humans and not rodents. For instance, the molecular interactions characterising cytoadhesion of IE to the vascular endothelium as observed in CM is not conserved between rodent species and humans. Thus, the contribution from animal models to our current understanding of human malaria is heavily questioned (reviewed in El-Assaad *et al*., 2014; Craig *et al*., 2012; White *et al*., 2010).

In the absence of appropriate experimental animal models, *in vitro* models are relied on for probing IE and endothelial cell interactions. *In vitro* models include cells derived from non-human origin such as transfected COS or CHO cells (Hasler *et al*., 1993; Berendt *et al*., 1989; Oquendo *et al*., 1989), and human cells including melanoma cells (C32) and primary (human dermal microvascular endothelial cells) or immortalised cells [human brain endothelial cells , HBEC5i] (Wassmer *et al*., 2006; Swerlick *et al*., 1992; Dorovini-Zis *et al*., 1991; Schmidt *et al*., 1982; Udeinya *et al*., 1981). Although these routinely used *in vitro* models for cytoadhesion have provided incredible information on host-parasite interactions, they may not reflect *in vivo* interactions (Andrews *et al*., 2005). Recently, Wassmer *et al*. (2011) isolated endothelial cells from subcutaneous fat tissue with comparable characteristics to brain endothelial cells. Although, this may represent a relevant model to study

cytoadhesion, the process of obtaining fat tissues may have ethical issues. The procedure appears to be delicate, time consuming, undesirable and hence cannot be routinely carried out. Clearly, more *in vitro* models are required to gain an in-depth understanding of disease mechanisms which will open up more possibilities for development of interventions.

Blood outgrowth endothelial cells (BOECs), also known as circulating endothelial colony forming cells (ECFCs) represent an excellent model to study parasite binding characteristics since they are an easily accessible source of material with specific endothelial lineage. BOECs are differentiated matured endothelial cells which are a subset of mononuclear cells, present in extremely low quantities (0.010 %) in healthy people (Dauwe *et al*., 2016; Timmermans *et al*., 2007; Yoder *et al*., 2007; Ingram *et al*., 2005) and the numbers appear to be altered in some disease states demonstrating the extent of endothelial damage. Notably, circulating endothelial cells are markedly elevated in sickle cell disease and the endothelium has been shown to be activated in the crisis state (Sakamoto *et al*., 2013; Strijbos *et al*., 2009; Solovey *et al*., 1997). BOECs can be derived from peripheral blood or cord blood with very high growth capacity when expanded in *in vitro* cultures and they are believed to be extremely suitable for angiogenesis cell therapy or vascular regeneration (Dauwe *et al*., 2016; Martin-Ramirez *et al*., 2012; Timmermans *et al*., 2007; Hur *et al*., 2004; Ingram *et al*., 2004). BOECs are characterised by utilising different culture techniques and or a combination of endothelial cell surface molecules. Specifically, they have a distinct endothelial cobblestone morphology in cultures, express most of the known endothelial cell markers such as CD34, CD146, von Willebrand factor (vWF) and platelet endothelial cell adhesion molecule 1 (PECAM-1; CD31), are negative for the progenitor cell marker CD133 and are capable of taking up acetylated low density

lipoproteins (Lin *et al*., 2000). BOECs provide an invaluable system or tool to study pathological diseases involving endothelial perturbation and investigating differences in the transcription levels of endothelial genes (Chang *et al*., 2008; Fernandez *et al*., 2005). Indeed, BOECs have been successfully isolated from patients with several types or isoforms of von Willebrand disease in order to assess the storage as well as secretion of vWF (Wang *et al*., 2013b). *Plasmodium falciparum* infection represents a disease where the vascular endothelium is prominently involved, serving as a site for IE sequestration. BOEC provides an alternative *in vitro* model for studying the endothelial-related pathology of human malaria since it has the capacity to express a potentially diverse range of cytoadhesion receptors.

CHAPTER THREE

THE ROLE OF AN ICAM-1 BINDING MOTIF IN PFEMP1 IN THE PATHOGENESIS OF CM

3.1 Introduction

The remarkable variations in the disease manifestations of individuals with *P. falciparum* infections even under similar levels of exposure have long been recognised (Miller *et al*., 2002; Greenwood *et al*., 1991). Specifically, most individuals resident in malaria endemic populations are either asymptomatic or experience mild disease which can be effectively managed. However, a minor proportion of individuals predominantly children, unfortunately succumb to life-threatening and fatal forms of the disease that usually presents as CM, respiratory distress and SA (WHO, 2016; Storm and Craig, 2014; Miller *et al*., 2013). SM kills about 400,000 individuals annually with most deaths markedly concentrated in children under five years (WHO, 2016). CM represents the most deadly SM complication with a high fatality rate of about 20 % even after compliance to prescribed treatment regimen. Even more, approximately one-third of survivours have variable levels of neuro-cognitive impediments (reviewed in Birbeck *et al*., 2010; Idro *et al*., 2010).

During the life cycle of *P. falciparum*, only early stage ring parasites are seen circulating in the peripheral blood; while mature stage parasites are withdrawn from circulation and preferentially sequester in the microvasculature in order to avoid splenic destruction (reviewed in Miller *et al*., 2002). Sequestration is believed to be the central precipitating event underlying CM pathogenesis as post-mortem studies provide evidence of extensive accumulation of IEs in brain vessels (Seydel *et al*., 2015; Milner, Jr. *et al*., 2014; MacPherson *et al*., 1985). Variant proteins exported to the surface of IEs are believed to promote sequestration by mediating adhesion to

diverse receptors displayed on endothelial cells which result in the blockage of blood flow, inflammation and damage to the endothelial barrier integrity (reviewed in Storm and Craig, 2014; Miller *et al*., 2002). Majority of the evidence gathered so far implicates the highly polymorphic parasite-derived protein PfEMP1, which are concentrated in knob-like projections on the surface of IEs (Howard *et al*., 1988; Leech *et al*., 1984a; Leech *et al*., 1984b). They are large proteins with variable molecular weights between 200-350 kDa and are encoded by approximately 60 different *var* genes which are expressed in a mutually exclusive pattern (Baruch *et al*., 1995; Smith *et al*., 1995; Su *et al*., 1995; Howard *et al*., 1988). Depending on their chromosomal location, upstream sequences and transcriptional orientation, PfEMP1 encoding genes are generally assigned into groups A, B, C or any one of two two hybrid groups of B/A and B/C (reviewed in Hviid and Jensen, 2015; Kraemer and Smith, 2003; Lavstsen *et al*., 2003). All PfEMP1 have a similar organisational structure comprising variable cytoadhesion domains of Duffy-binding like proteins (DBL) and cysteine rich inter-domain region proteins (CIDR) that are responsible for binding host receptors. Within the DBL and CIDR domains, subgroupings based on sequence identity have been identified and these are believed to be associated with specific receptor interactions (Rask *et al*., 2010; Smith *et al*., 2000). Notably, CIDRα2-6 domains of groups B and C frequently adhere to CD36 (Hsieh *et al*., 2016; Robinson *et al*., 2003), while CIDRα1 of groups A and B PfEMP1 variants interact with EPCR (Lau *et al*., 2015; Turner *et al*., 2013).

A fundamental question in malaria pathogenesis has always been whether a specific binding phenotype can be linked to any of different SM phenotypes given the observation that different receptors have been implicated in PfEMP1 binding. The enormous sequence diversity found within PfEMP1 molecules has frustrated efforts in

identifying such conserved functional regions. Nevertheless, the discovery that a unique PfEMP1 variant called VAR2CSA specifically interacts with placental CSA during PAM (Salanti *et al*., 2004; Fried and Duffy, 1996) appears to suggest the existence of some conserved PfEMP1 determinants involved in organ-specific severe disease. This striking example for PAM has spurred the continued search for restricted PfEMP1 variants responsible for other severe disease complications including CM. Indeed, many studies have shown that parasites expressing group A and B/A PfEMP1 that mediate EPCR binding are associated with SM syndromes (Bertin *et al*., 2013; Turner *et al*., 2013; Lavstsen *et al*., 2012). More importantly, the molecular details of the CIDRα subtype within PfEMP1 binding to EPCR were described based on sequence information (Lau *et al*., 2015). Altogether, these findings have heightened the interest of potentially identifying specific binding phenotypes connected with CM development, the most lethal form of severe disease.

Earlier reports have suggested ICAM-1 as a receptor for sequestration of IEs during CM pathogenesis (Chakravorty and Craig, 2005; Kaul *et al*., 1998; Turner *et al*., 1994). Immunohistochemical assessment of autopsy specimens of CM provides evidence of increased endothelial ICAM-1 expression together with retained IEs in the brain microvasculature (Taylor *et al*., 2004; Silamut *et al*., 1999; Turner *et al*., 1994). However, the role of ICAM-1 in CM has not always been clear-cut. Indeed, the ability to bind ICAM-1 has been found both in DBLβ domains of groups B and C PfEMP1 associated with UM, and group A DBLβ domains linked to CM (Bengtsson *et al*., 2013b; Janes *et al*., 2011; Howell *et al*., 2008). Additionally, *in vitro* selection of IEs on human brain endothelial cells expressing PfEMP1 of DC8 and DC13 adhered to EPCR and not ICAM-1 (Turner *et al*., 2013). Altogether, this indicates that ICAM-1 binding may not be sufficient to initiate CM pathogenesis.

In 2013, Bengtsson *et al*., aimed to understand the molecular underpinnings of group A PfEMP1 associated with ICAM-1 binding in CM. The study searched for orthologous sequences of the group A PFD1235w *var* gene which is thought to mediate ICAM-1 binding and has been previously associated with the development of SM (Jensen *et al*., 2004). By using PFD1235w-specific primers and a degenerate group A exon 2–specific primer, *pfd1235w*-like genes were amplified from parasite isolates obtained from Ghanaian children with *falciparum* malaria. The PFD1235w-like *var* gene-encoded form of PfEMP1 obtained from the parasite isolates possessed highly identical three tandem domain (DBLα1.1/1.4-CIDRα1.6-DBLβ3) sequences located N-terminally and was called domain cassette four (DC4) [Figure 3.1].

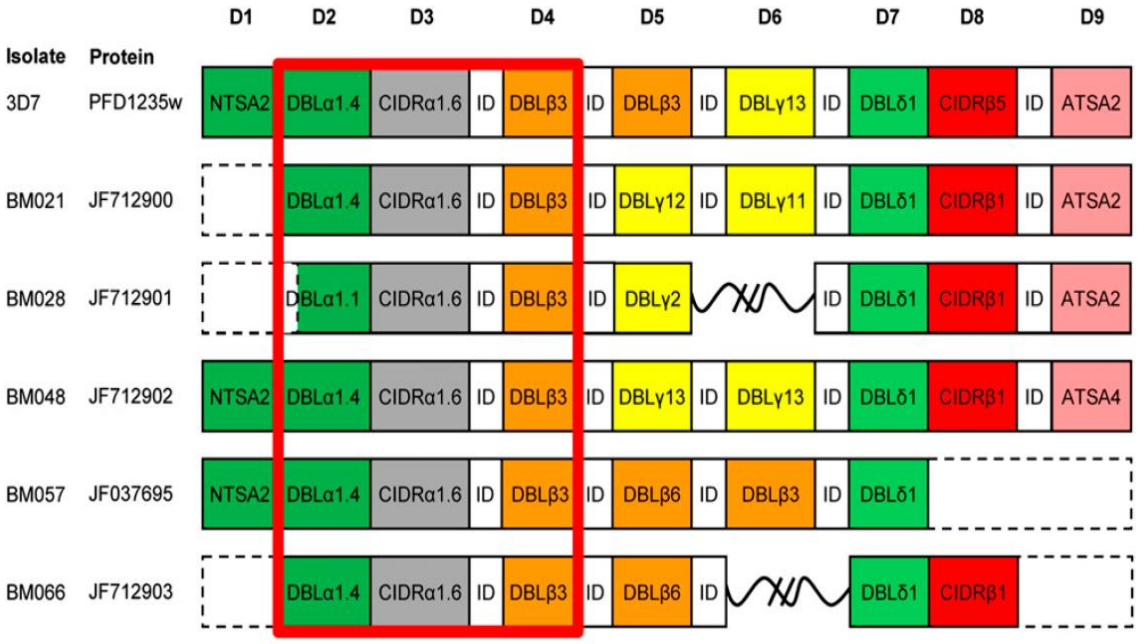


Figure 3.1: Domain structural organisation of PfEMP1 proteins containing DC4. The PfEMP1 proteins consist of the 3D7 PFD1235w and five PfEMP1 isolated from Ghanaian f*alciparum* parasites. The DC4 protein in defined by three tandem domains of DBLα1.4/DBLα1.1-CIDRα1.6-DBLβ3 and it is enclosed in the red rectangle. A high sequence identity is observed within DC4 among the parasite isolates in comparison with domains outside DC4 (Bengtsson *et al*., 2013b).

The domain involved in ICAM-1 binding was mapped to the DBLβ3 domain four (DBLβ3_D4) and not DBLβ3 domain five (DBLβ3_D5) which is located outside the DC4 region (Figure 3.1). Indeed, up to 80 % sequence similarity was observed among DBLβ3_4 domain of the different parasite isolates. Furthermore, a sequence analysis showed that DBLβ3 of DC4 formed a tight phylogenetic cluster which was different from other ICAM-1 binding and non-binding DBLβ domains (Figure 3.2).

IEs expressing DC4 were found to mediate ICAM-1 binding. Importantly, naturally-acquired DC4 DBLβ3 antibodies were cross-reactive and cross-inhibitory to DC4-containing ICAM-1 binding IEs and to a non-DC4, group A DBLβ1 from Dd2VAR32 [DC13] (Bengtsson *et al*., 2013b). To locate the exact ICAM-1 binding site or region within the DBLβ3_4, hybrid proteins were expressed by domain swapping. Notably, the proteins consisted of possible combinations of the extracellular portions of the ICAM-1 binding DC4 DBLβ3_D4 and a non-ICAM-1-binding DBLβ3_D5 of 3D7 PFD1235w. Hybrid proteins harbouring the C-terminal portions (4C region) of DBLβ3_D4 maintained the ICAM-1 binding property (Figure 3.3) and also DBLβ3 ICAM-1 inhibitory antibodies were able to specifically target this region.

The aim of this present study was to dissect further the molecular details characterising ICAM-1 binding by group A PfEMP1 by using sequence information to define a sequence motif that can be used to identify ICAM-1-binding DBLβ domains. Additionally, the relationship between the expression of DBLβ domains involved in ICAM-1 binding and CM development was investigated.

This study formed part of a collaborative research with Professor Matthew Higgins' laboratory (Oxford University) where the structural biology experiments were done

and the data presented in this chapter have been published recently in the Cell Host and Microbe Journal (Lennartz *et al*., 2017; Appendix E).

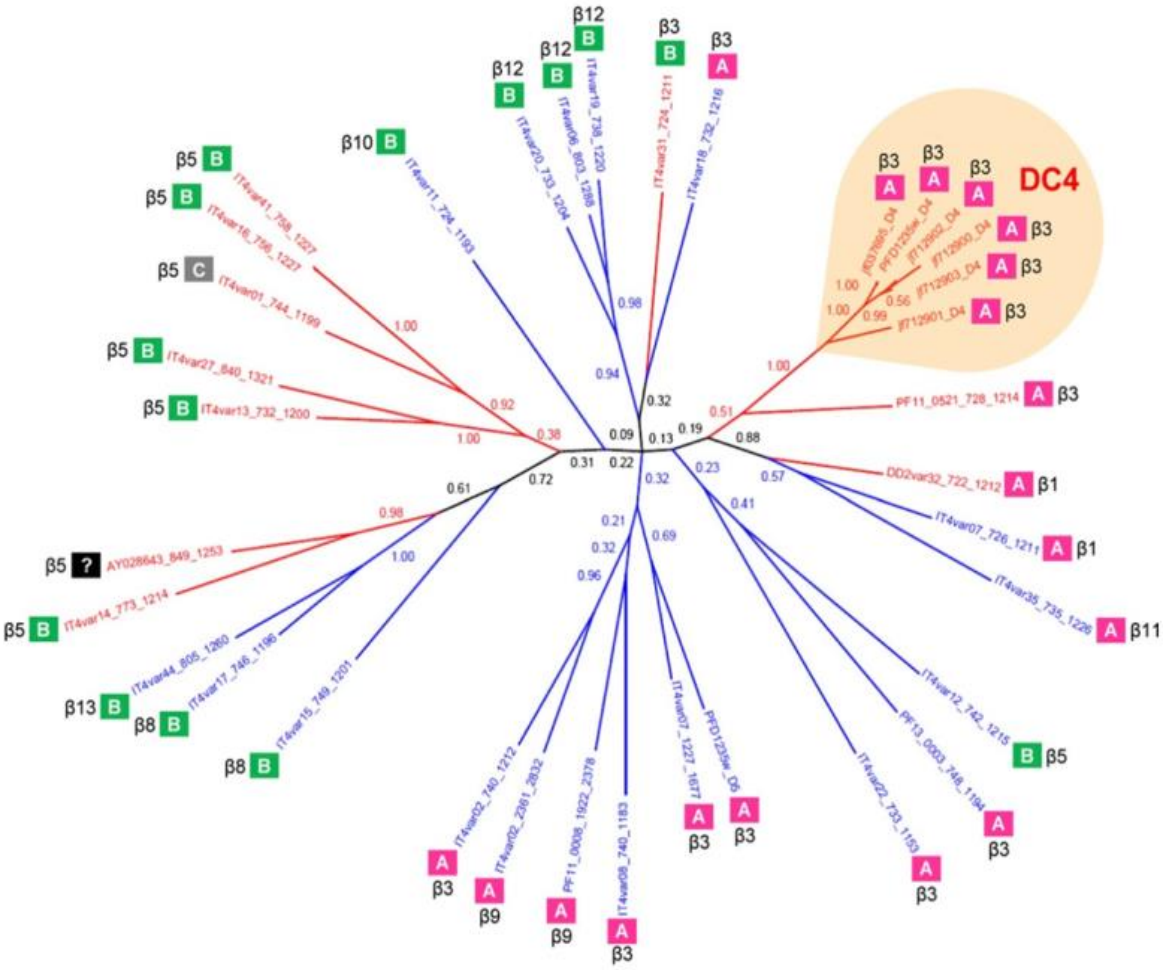


Figure 3.2: Phylogenetic tree of 16 ICAM-1 binding (red) and 19 non-ICAM-1 binding (blue) DBLβ domains. The round shaded area represents DBLβ domains present in DC4. ´A, B, C' represent the *var* groups in which the DBLβ can be found (Bengtsson *et al*., 2013b).

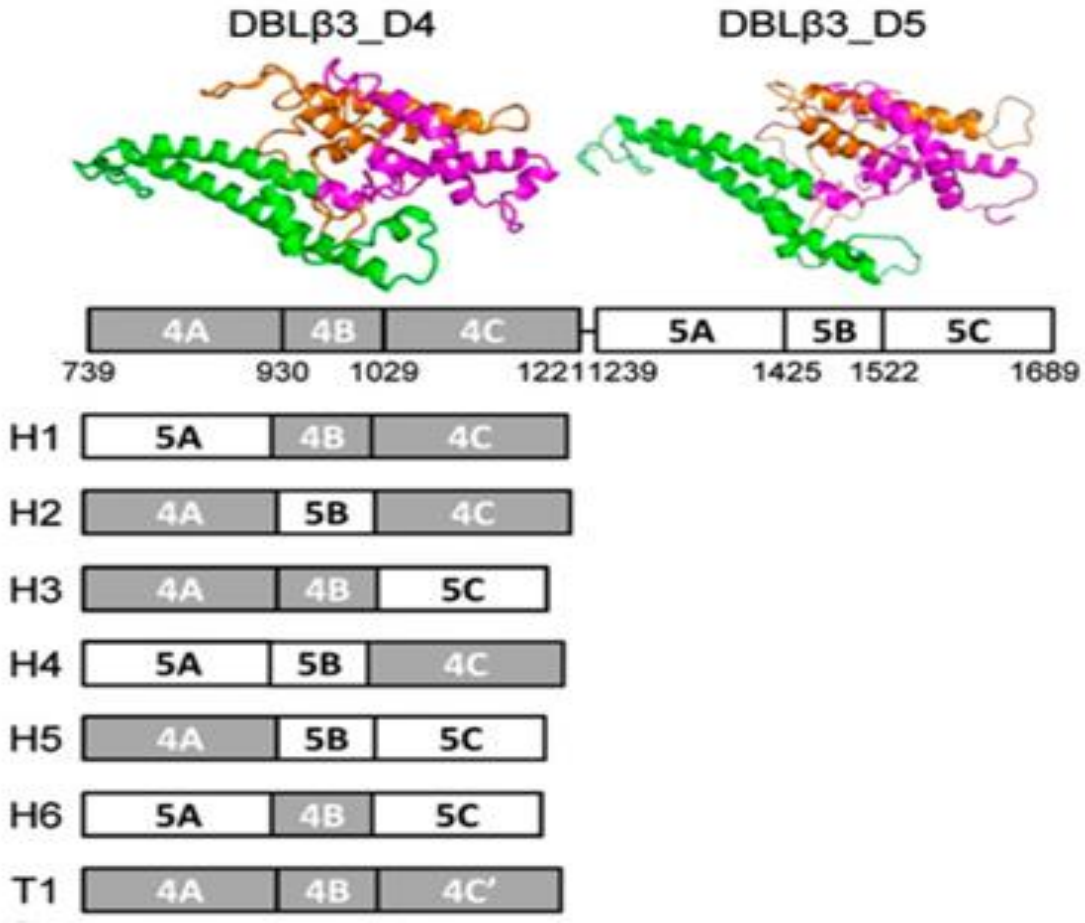


Figure 3.3: Identification of the ICAM-1 binding region in PFD1235w DBLβ3_D4. A model of the PFD1235w domains of DBLβ3_D4 and DBLβ3_D5 showing the extracellular portions (green, magenta, pink). Schematic diagrams of PFD1235w domains DBLβ3_D4 (gray), DBLβ3_D5 (white) and hybrids (H1–H6) of the extracellular portions (A-C). T1 represents a DBLβ3_D4 shortened at the C-terminal side. ICAM-1 binding was localised to the 4C region of DBLβ3_D4 (Bengtsson *et al*., 2013b).

3.2 Hypothesis and specific objectives

Plasmodium falciparum IEs associated with CM express a particular subset of group A PfEMP1 that bind to ICAM-1 and EPCR. Such PfEMP1 contain a specific amino acid sequence (motif) in the DBLβ domain that mediates the ICAM-1 binding.

Specific objectives

1. To evaluate the binding interactions between ICAM-1 and recombinant group A PfEMP1 DBLβ domains

2. To measure motif-specific antibody responses in plasma samples from individuals living in malaria endemic regions
3. To compare the transcription levels of motif-encoding *var* genes and EPCR binding CIDRα domains in defined categories of malaria patients
4. To determine the binding of IE expressing PfEMP1 containing the motif to ICAM-1 and EPCR under flow conditions

3.3 Methods

3.3.1 Study sites, ethics and sample collection

The study cohorts were recruited from Hohoe Municipal Hospital in the Volta Region of Ghana (Kweku *et al*., 2008), from Korogwe District Hospital in the Korogwe District of Northeastern Tanzania (Lavstsen *et al*., 2012), and from the Centre National Hospitalier Universitaire Hubert Koutoucou Mega in Cotonou, Benin (Moussiliou *et al*., 2015). The study sites are characterised by marked differences in the intensity of *P. falciparum* malaria transmission and these were selected to increase the possibility of acquiring enough parasite isolates. The ethics and study protocols as well as permit to conduct the study were reviewed and approved by the Institutional Review Board of the Noguchi Memorial Institute for Medical Research, Ghana (Study number 026/13-14), the Ethical Review Committee of the Ghana Health Service (MOH; file GHS-ERC 08/05/14; Appendix A), the Ethical Review Board of the National Institute for Medical Research, Tanzania (National Institute of Medical Research/HQ/R.8a/Vol.IX/559) and by the Ethics Committee of the Research Institute of Applied Biomedical Sciences, Cotonou, Benin (N°21/CER/ISBA/13). Patients were only enrolled if their parents or guardians voluntarily consented to partake after the

goals of the study were explained and also if they met the study requirements. Study participants could exit from the study voluntarily without any consequences. Following informed consent, children younger than 12 years of age with differential SM clinical syndromes were enrolled in the study and underwent clinical investigations.

Thick and thin blood smears were prepared on the same microscope slide from the blood collected. An aliquot of blood (7 μL) was placed at one end of a microscope slide and evenly spread out in a circle of approximately 1-2 cm diameter using one corner of a clean microscope slide to make the thick smear. Approximately 2 μL of blood was placed on the same microscope slide with the thick smear (almost in the mid portion). A second microscope slide was used to spread the blood across the slide quickly but gently to produce a thin film. The blood smear was air dried, fixed for 10 s in 100 % methanol (VWR, USA) and stained for 15 min with 20 % Giemsa (Merck, Germany) diluted in Giemsa buffer pH 7.2. The slide was gently rinsed with water, air dried and then examined under oil with the 100 X objective of a light microscope. Parasitaemia was estimated as parasites per 200 white blood cells. Patients who consented were grouped according to the clinical definitions outlined by the WHO on clinical and laboratory criteria (WHO, 2000). Patients with CM were defined as being microscopy positive for *P. falciparum* infection with a Blantyre coma score (BCS) ≤ 2, attributed to *falciparum* infections only. SA patients were defined as having haemoglobin (Hb) < 5g/dL and BCS >2 in addition to infection with *falciparum*. Other malaria patients were defined as having BCS >2 and Hb levels >5 g/dL with detectable peripheral *falciparum* parasites.

Plasma samples from malaria-exposed Tanzanian individuals (1-17 years) involved in a previous study investigating risk factors for anaemia and febrile malaria (Lusingu *et*

al., 2004) and also nine plasma samples from immune Liberian adults from another previous study (Theisen *et al*., 2000) were additional samples used in this study to assess the acquisition of antibody responses to identified motif in individuals from different malaria transmission settings

3.3.2 Sample processing

Processing of blood samples was carried out under aseptic conditions and all work was done in a microbiological biosafety II cabinet which was regularly cleaned with detergent and 70 % ethanol. Blood samples were centrifuged (800 x g; 5 min) to separate plasma samples. The pellet which contained IEs was washed in RPMI 1640 with 25 mM Hepes modified (Sigma-Aldrich, Germany), supplemented with 50 µg/mL gentamicin sulfate (Sigma-Aldrich, Germany), [referred to as incomplete RPMI] and centrifuged (500 x g; 5 min). The wash process was repeated once. The washed pelleted erythrocytes containing parasites was resuspended in Glycerolyte 57 freezing medium (Fenwal, USA) in a ratio of five volumes of freezing medium to three volumes of cell pellet in two steps. Firstly, one volume of freezing medium was added in dropwise with gentle swirling and the mix was allowed to stand for 5 min before slow addition of the remaining four volumes. The resulting suspension was gently mixed, aliquoted into cryovials (1.0 mL per cryovial) and placed in Mr. Frosty freeze containers to allow slow freezing at -80 ^{0}C overnight before being transferred to liquid nitrogen for long-term storage. A portion of the washed pelleted IEs was stored in TRIzol reagent (Thermofisher Scientific, USA) for purification of RNA to assess *var* gene transcript levels. Briefly, TRIzol reagent (Thermofisher Scientific, USA) was allowed to equilibrate to room temperature (room temp) and about 50-200 µL of the pelleted erythrocytes was completely dissolved in nine volumes of TRIzol

reagent by pipetting several times to uniformly mix the pellet. The resulting mix was finally vortexed to obtain a dark brown suspension with no traces of clumps and was stored at -80 ^{0}C freezer until use.

3.3.3 Recombinant proteins

Recombinant DBLβ domains containing the ICAM-1 motif or non-motif DBLβ proteins and a head structure of PFD1235w (CIDRα1.6- DBLβ3) were produced in *E. coli* SHuffle C3030 cells [New England Biolabs, UK] (Bengtsson *et al*., 2013b). Briefly, these proteins were produced from genomic DNA amplified by PCR using published primers (Appendix B).The PCR products were sub-cloned into a modified pET15b vector and expressed as N-terminal, hexahistidine-tagged (his-tagged) proteins in *E. coli* SHuffle C3030 cells (New England Biolabs, UK). A hybrid of PFD1235w DBLβ3_D4 (amino acid 739-1,221) and PFD1235w DBLβ3_D5 (amino acid 1,239-1,689) termed D5_motif were cloned by overlapping PCR amplification and expressed in *E. coli* SHuffle C3030 cells. The expressed proteins were purified by immobilised metal ion affinity chromatography using HisTrap^{TM} HP 1 mL column (GE Healthcare, Denmark). In addition to these proteins, previously produced DBLβ proteins (Table 3.1) were also used (Bengtsson *et al*., 2013b). The motif peptide (LYAKARIVASNGGPGYYNTEVQKKDRSVYDFLYELHLQNGGKKGPPPATHP YKSVNTRDKRDATDDTTP) derived from PFD1235w DBLβ_D4 was produced by Schaefer-N, Denmark. Human ICAM-1 combined with the Fc region of human IgG1 (ICAM-1-Fc) was cloned, expressed as His-tagged protein in HEK293-F cells (Invitrogen, USA) according to the manufacturer's instructions and described in (Bengtsson *et al*., 2013a) was previously produced. The recombinant protein was purified from the supernatant on a HiTrap Protein G High Performance column (GE

Healthcare, Denmark), eluted using 0.2 M glycine/HCl buffer (pH 2.5), neutralised in suspension using Tris/ HCl buffer (1 M, pH 9) and finally buffer-exchanged into phosphate buffered saline (PBS) (Bengtsson *et al*., 2013b).

3.3.4 Rat immunisations

All procedures concerning animal experiments and immunisations complied with the Danish national regulations. The animal procedures described in this study were carried out according to the guidelines elaborated in act numbers LBK 1306 (23/11/2007) and BEK 1273 (12/12/2005). Permission to carry out experiments was granted by the Danish Animal Procedures Committee ("Dyreforsøgstilsynet") and detailed in permit number 2008/561-1498. Before immunisation, sera was collected from each animal and used in flow cytometry and binding assays as negative controls. Recombinant PfEMP1 DBLβ constructs (3D7 PFD1235w_D4 [new ID: PF3D7_0425800_D4]; and IT4VAR13 (new ID: ABM88750) and synthetic peptide, (LYAKARIVASNGGPGYYNTEVQKKDRSVYDFLYELHLQNGGKKGPPPATHP YKSVNTRDKRDATDDTTP) [Schaefer, Denmark] from 3D7 PFD1235w_motif were used to immunise rats (Wistar; 8-12 weeks of age; three animals per immunisation group) to generate rat antisera. Procedures to generate rat antisera preparations have been previously described (Bengtsson *et al*., 2013b). Briefly, rats were subcutaneously injected with 25 µg of protein in Freund's incomplete adjuvant followed by two booster vaccinations of 15 µg in incomplete Freund's adjuvant. Post-immunisation sera were taken two weeks following the last immunisation.

3.3.5 Antibody purification

Rat IgG specific for DBLβ proteins and the motif (anti-PFD1235w_D4; anti-PFD1235w_motif; anti-IT4VAR13) were purified from rat antisera using previous procedures (Joergensen *et al*., 2010). Briefly, 1 mg of protein in each 1 mL was dialysed overnight against coupling buffer and further coupled to Hi-Trap NHS-activated High Performance columns following the manufacturer's protocol (GE Healthcare, Denmark). Antibodies were diluted (1:1 in PBS), affinity purified, eluted in glycine buffer (0.1 M, pH 2.75) and neutralised in 1M Tris/HCl (pH 9.0). Pre-immunisation serum or rat IgG (Sigma-Aldrich, Germany) was used as negative controls in binding assays. Pooled plasma from nine immune Liberian adults (Theisen *et al*., 2000) was used for affinity purification of DBLβ and motif specific human IgG (anti-PFD1235w_D4; anti- PFD1235w_motif; anti-IT4VAR13). Human IgG (Sigma-Aldrich, Germany) was used as negative control in binding assays.

3.3.6 ELISA-based binding inhibition assay

ICAM-1 binding to different recombinant DBLβ proteins was measured by ELISA as previously described before (Bengtsson *et al*., 2013b). Maxisorp plates (Nunc, Thermofisher Scientific, Denmark) were coated with different recombinant DBLβ domains (50 µL; 2-10 µg/mL; 0.1 M glycine/HCl buffer pH 2.75) overnight at 4^0C and blocked with blocking buffer (PBS, 0.5 M NaCl, 1 % Triton-X-100, 1 % BSA, 0.03 mM phenol red, pH 7.2; 1 h; room temp). The plates were emptied and washed four times in PBS-Triton-X-100 (PBS, 0.5 M NaCl, 1 % Triton X-100, pH 7.4). Recombinant ICAM-1–Fc chimera (0.5-16 µg/mL; 50 µL) was added to duplicate wells and incubated (1 h; room temp). Following washing, bound chimera was detected with rabbit anti-human IgG-HRP (Dako, Denmark; 1:3,000) targeting the Fc-

portion of the ICAM-1-Fc. A total of 26 recombinant DBLβ proteins were screened for ICAM-1 binding.

Similar to the protocol described above motif-specific IgG reactivity was assayed in plasma samples collected from immune adults. Plates were coated with motif containing DBLβ (50 µL; 2 or 4 µg/mL; 0.1 M glycine/HCl buffer pH 2.75; overnight at 4 °C) and blocked with blocking buffer (1h; room temp). Diluted plasma (1:100) were incubated (1 h; room temp) and bound human IgG was detected using rabbit anti-human IgG (Dako, Denmark; 1:3000; in blocking buffer; 50 µL).

Inhibition of ICAM-1 binding to DBLβ domains by rat antisera was performed using a modified version of the ELISA protocol. Briefly, plates were coated with ICAM-1 (50 µL; 2 or 4 µg/mL ICAM-1; 0.1 M glycine/HCl buffer pH 2.75; overnight at 4 °C) and blocked with blocking buffer (1h; room temp). His-tagged recombinant DBLβ domains (0.5-16 µg/mL) were mixed with affinity purified antisera (1:5) or purified human IgG (0.25-32 µg/mL) and subsequently incubated (1 h; room temp) with pre-coated ICAM-1 plates. Following washing, bound DBLβ was detected with Penta-His antibody conjugated to HRP (Qiagen, UK; 1:3,000). All antisera were initially assessed for His-tag specific antibodies by ELISA and only sera that were negative for His-tag reactivity were included in the assay.

3.3.7 RNA purification and DNase treatment

This work was carried out in the fume hood due to toxic fumes from chloroform and TRIzol. RNA is very sensitive to ubiquitous RNases, thus tubes and tips that have not been in contact with skin were used and “RNase Away” spray was used to clean pipettes. Gloves were won throughout the whole purification period and were changed frequently to prevent contamination. RNA was extracted from both field isolates and

selected ring stage laboratory parasite isolates (see section 3.3.13). Frozen TRIzol samples were thawed to room temp, 500 µL of thawed sample was pipetted into a 1.5 mL microcentrifuge tube and 500 µL of TRIzol (Thermofisher Scientific, USA) added. Two hundred microlitres of chloroform (Sigma-Aldrich, Germany) was added, the suspension was shaken vigorously for about 15 s, allowed to sit at room temp for 2-3 min and centrifuged (12,000 x g; 15 min; 4^0C). The aqueous phase was collected, and 500 µL of isopropanol (Sigma-Aldrich, Germany) and 5 µL glycogen (Thermofisher Scientific, USA) were added. The suspension was mixed a few times, allowed to stand for 10 min at room temp and then centrifuged at 12,000 g for 10 min at 4 ^{0}C to pellet RNA. The supernatant was gently decanted, the pellet washed in 1 mL of 75 % ethanol in 0.1 % diethylpyrocarbonate in deionised water (DEPC-H_20), prior to centrifugation (7500 x g; 5 min; 4 ^{0}C). The supernatant was gently aspirated without disturbing the pellet and dried (7-10 min; room temp). Dried samples were resuspended in 12 µL of DEPC-H_20, heated at 65 ^{0}C for 5 min and quickly placed on ice.

Extracted RNA was immediately treated to remove genomic DNA contamination. DNase treatment was done according to the manufacturer by adding 2 µL DNase1 (Sigma-Aldrich, Germany), 1.5 µL of 10X DNase buffer (Sigma-Aldrich, Germany) to 11.5 µL of extracted RNA. Following incubation (20 min; 37 ^{0}C), 2 µL of stop solution (Sigma-Aldrich, Germany) was added and then incubated again (70 ^{0}C; 15 min) to inactivate the enzyme. The treated RNA was stored at -80 ^{0}C freezer or processed for confirmation of the absence of DNA in RNA samples by quantitative real-time polymerase chain reaction (qPCR) with *seryl-tRNA synthetase* primers (p90) (Salanti *et al*., 2003). Briefly, 0.5 µL of RNA was added to 0.2 mL PCR tube containing 10 µL of SYBR Green PCR mastermix (Qiagen, UK), 8 µL of DEPC-H_20

and 2 µM of p90. qPCR was carried out using a Rotorgene thermal cycler system (Corbett Research, UK) with the following cycling conditions: 95 C for 15 min, followed by 40 cycles of 95 ^{0}C for 30 s, 50 ^{0}C for 40 s, and 65 ^{0}C for 50 s, with final elongation at 68 ^{0}C for 40 s.

3.3.8 cDNA synthesis and qPCR

Samples free of genomic DNA contamination were reverse transcribed to obtain cDNA. For the synthesis of first strand cDNA, 15 µL of RNA was placed in 0.2 µL PCR tube and 0.02 µg/µL of random primers (Thermofisher Scientific, USA), as well as 1.74 µM Deoxynucleotide Triphosphates mix (Invitrogen, USA) were added. The reaction mix was vortexed, briefly span, incubated for 10 min at 65 ^{0}C and placed on ice immediately. To the reaction tube, 6 µL of 10x first strand buffer (Thermofisher Scientific, USA), 3 µL of dithiothreitol (Thermofisher Scientific, USA), 1 µL of RNaseOUT recombinant enzyme (Thermofisher Scientific, USA) and 1 µL of Superscript II enzyme (Thermofisher Scientific, USA) were added. The reaction mixture was vortexed and briefly centrifuged. cDNA synthesis was carried out in a PCR machine (MWG-Biotech, Germany) and synthesis settings used were incubation at 25 ^{0}C for 10 min, 42 ^{0}C for 50 min, 70 ^{0}C for 15 min and 4 ^{0}C for a minimum of 4 min. cDNA was stored at -20 ^{0}C until use for *var* transcript analysis. cDNA was tested for absence of contaminating genomic DNA by qPCR with p90 primers (Salanti *et al*., 2003) as described above for the absence of contaminating DNA in purified RNA samples.

Primers specific for *var* gene sub-types encoding group A DBLβ domains predicted to bind to ICAM-1 (motif primers: Q183/Q186) and/or *var* gene sub-types encoding CIDRα1 domains involved in EPCR-binding ("DBLα2/1.1/2/4/7") (Mkumbaye *et al*.,

2017) (Appendix B) were used to amplify these *var* gene groups from cDNA. For laboratory isolates, *var* gene specific primers for each of the *var* gene present in 3D7 (Dahlback *et al*., 2007; Salanti *et al*., 2003), HB3 (Soerli *et al*., 2009) and IT4 (Wang *et al*., 2012) were used. qPCR was performed in 20-µL reaction mix using QuantiTect SYBR Green PCR master mix (Qiagen, UK) on a Rotorgene thermal cycler system (Corbett Research, UK). The amplification cocktail included 0.2 µL of cDNA, 10 µL of Quantitect SYBR Green PCR Master Mix (Qiagen, UK) and 2 µM primers. qPCR cycling conditions were 95 ^{0}C for 15 min, followed by 40 cycles of 95 ^{0}C for 30 s, 50 ^{0}C for 40 s, and 65 ^{0}C for 50 s, with final elongation at 68^{0}C for 40 s. Data were acquired at the end of elongation of each cycle and the specificity of amplifications was assessed by melting-curve analysis. The cycle threshold (Ct) was set at 0.025. The transcript abundance was determined relative to the average transcript abundance of the housekeeping gene *seryl-tRNA synthetase* using the formular: $\Delta Ct_{var\_primer} = Ct_{var\_primer} - Ct_{average\_control\ primers}$ as previously described (Dahlback *et al*., 2007). ΔCt_{var_primer} values were converted into Transcript Units ($Tu = 2^{(5-\Delta Ct)}$) as described previously (Lavstsen *et al*., 2012). Briefly, *var* transcript levels with a Ct value of 5 or higher than the average of the endogenous housekeeping gene Ct value was given a value of 5 and the Tu calculated as $2(5- \Delta Ct_{var\_gene})$. Notably, Tu value of 1 was given to ΔCt_{var_gene} values ≥ 5. $\Delta Ct_{var\_primer} = 4$ is equivalent to Tu = 2, $\Delta Ct = 3 \approx Tu = 4$, $\Delta Ct = 2 \approx Tu = 8$, $\Delta Ct = 1 \approx Tu = 16$, $\Delta Ct = 0 \approx Tu = 32$ which is equivalent to the average value of the transcription of the endogenous housekeeping gene.

3.3.9 Parasite cultures

Culturing of parasites was carried out under aseptic conditions and all work was done in a microbiological biosafety II cabinet which was routinely cleaned with detergent

and 70 % ethanol. The well-established laboratory *P. falciparum* clonal lines 3D7, HB3 and IT4 were used in this study. The HB3 isolate was originally obtained from Honduras (Bhasin and Trager, 1984), 3D7, a clone from NF54 which may possibly be of African origin (Walliker *et al*., 1987) and IT4 which was isolated from Brazil (Udeinya *et al*., 1983). These isolates (3D7, HB3 and IT4) were maintained for long-term *in vitro* and selected for IE surface expression of specific PfEMP1 (Bengtsson *et al*., 2013b; Joergensen *et al*., 2010). *Plasmodium falciparum* parasites were grown in sterile flasks of 25 cm^2 or 75 cm^2 (Thermofisher Scientific, Denmark) and cultured using a well-described method (Cranmer *et al*., 1997) with slight modification. Briefly, parasites were maintained in RPMI 1640 with 25 mM Hepes modified (Sigma-Aldrich, Germany), 50 µg/mL gentamicin sulphate (Sigma-Aldrich, Germany), 4 mM Glutamine (Sigma-Aldrich, Germany), 0.5 % Albumax II (Invitrogen, USA), [now referred to as culture medium] and human erythrocytes (O+). Parasites in flasks were cultured in a gas mixture of 2.0 % O_2, 5.5 % CO_2, and 92.5 % N_2 (Strand Møllen, Denmark) and incubated at 37 ^{0}C. The culture medium was changed every other day with pre-warmed medium (37 ^{0}C) and Giemsa-stained smears were also made from 3 µL of parasite culture to estimate parasitaemia. Cultures were routinely maintained at 1 % parasitaemia by adding washed human erythrocytes, culture medium and gassed. Human erythrocytes (O+) used for culturing were obtained from the Copenhagen Hospital Biobank (Denmark), washed twice in incomplete RPMI and centrifuged (500 x g; 8 min) to remove plasma and leucocytes. Washed erythrocytes were resuspended at 50 % haematocrit in incomplete RPMI and stored at 4 ^{0}C for about two weeks. Mycoplasma infection was checked regularly using the MycoAlert Mycoplasma Detection Kit (Lonza, USA) according to the manufacturer's instructions. The genotype of parasites was verified by amplifying the

polymorphic proteins, merozoite surface 2 (MSP 2) and glutamate rich protein (GLURP) using standard methods (Snounou *et al*., 1999).

3.3.10 Thawing of frozen parasites

All solutions used for thawing were pre-warmed to 37 ^{0}C. The parasite stabilate was allowed to thaw rapidly at 37 ^{0}C after careful removal from the liquid nitrogen. Following thawing, the content was transferred to a 50 mL falcon tube and 200 µL 12 % NaCl (VWR, USA) was added to 1 mL of packed cell volume in dropwise with gentle swirling. After 5 min of incubation at room temperature (room temp), 10 mL of 1.6 % NaCl (VWR, USA) was added in drops, followed by another 10 mL of 0.9 % NaCl (VWR, USA) containing 0.2 % dextrose (Baxter, USA). The mixture was centrifuged (500 x g; 5 min), washed once in incomplete RPMI and resuspended in culture medium at 2 % haematocrit. The cell suspension was transferred to a 25 cm^2 culture flask, gassed for 30 s and incubated at 37 ^{0}C. The next day, parasitaemia was assessed and medium replaced with fresh culture medium.

3.3.11 Gelatin purification

This procedure separates mature stage trophozoite-IEs from the young and immature ring stages and uninfected erythrocytes, thereby synchronising the parasite culture. Briefly, cultures with predominant late stages (3-5 %) were centrifuged (500 x g; 5 min) and the supernatant carefully decanted. Pre-warmed gelatin (Sigma-Aldrich, Germany) [3 mL of 0.75 % in H_2O] was added to resuspend the parasite pellet and incubated (37 °C; 20 min) to enrich for late stages. Mature IEs in the top layer of the parasite enriched gelatin suspension were carefully transferred to a round bottom tube, washed twice in incomplete RPMI (10 mL) and centrifuged (500 x g; 5 min). The

purified mature IEs were used for antibody selection (laboratory parasites) of specific PfEMP1 expression.

3.3.12 Magnetic activated cell sorting (MACS) purification

Late trophozoites and schizonts (at least 2 %) were purified from uninfected erythrocytes and ring-stage IEs using MACS. This purification takes advantage of the accumulation of haemozoin, a degradative product of haemoglobin making late stage IEs susceptible to being captured by a strong magnet. The MACS column (Miltenyi Biotec, Germany) was fitted onto a magnetic stand; a 20 G needle and a 3-way stop were fitted onto the attached column. The MACS column was washed with 20 mL PBS supplemented with 2 % foetal bovine serum (Sigma-Aldrich, Germany) [2 % FBS] and to remove trapped air bubbles in the column. About 10 mL of 2 % FBS was allowed to run through the tap fitted onto column at a slow rate (about 2 drops per s). The parasite suspension (in 2 % FBS) was added to the column and allowed to flow through and the column was washed with 20 mL 2 % FBS until a clear run through was observed. A plunger was inserted on top of the column and a syringe filled with 15 mL of 2 % FBS was fitted onto the plunger. The column with the fitted syringe was removed from the magnet and the 2 % FBS in the syringe pushed forcibly through the column into a 50 mL falcon tube to elute the MACS-purified IEs. The syringe was filled again with 10 mL of 2 % FBS to elute any residual IEs still present in the column. The concentration of the cell suspension was enumerated on a Neubaeur haemocytometer and cells were used for IE surface reactivity flow cytometry assay or antibody specific selection.

3.3.13 Parasite selection for surface reactivity

Late stage *P. falciparum* clonal lines (3D7, HB3, IT4) were selected for IE surface expression of specific PfEMP1 (Bengtsson *et al*., 2013b; Joergensen *et al*., 2010). Briefly, 3D7 IEs were selected with the human monoclonal antibody AB01 (15 µg), which targets 3D7 PFD1235w DBLγ13_D6 (PF3D7_0425800_D6) (Barfod *et al*., 2011) or with a polyclonal depleted rat anti-serum against PF11_0008 CIDRβ4_D6 (1:20 in incomplete RPMI). *Plasmodium falciparum* IT4 IEs were selected using sterile filtered depleted rat anti-serum (1:20 incomplete RPMI) against IT4VAR13 DBLβ5_D4 (ABM88750_D4), while the selection of HB3 IEs was done using sterile filtered depleted rat anti-serum (1:20 in incomplete RPMI) against HB3VAR03 DBLβ3_D4 (new ID: KOB63865_D4).

For 3D7 selection, protein A coupled Dynabeads (25 µL; Invitrogen, USA) were aliquoted into 12 mL centrifuge tube and 8 mL of incomplete RPMI was added to wash the beads. The tube containing the beads was placed on a magnetic stand; the tube inverted a few times and then allowed to stand for about 1 min. The medium was aspirated and the wash process repeated. Sterile filtered AB01 (15 µg diluted in 1 mL incomplete RPMI) was added to the beads and incubated (30 min; room temp) on a rocking table. The tube containing the mixture was placed on a magnetic stand and the wash process repeated thrice to remove unbound antibodies. Gelatin purified 3D7 IEs were resuspended in 2 mL of incomplete RPMI and added to the AB01 coated beads and incubated (30 min; room temp) on a rocking table. For the 3D7 PF11_0008, HB3 or IT4 selection, gelatin-purified IEs resuspended in 2 mL of incomplete RPMI were added to sterile depleted rat antisera, incubated (30 min; room temp) and the unbound parasites removed by washing three times with 10 mL of incomplete RPMI, followed by centrifugation (500 x g, 5 min). Simultaneously, Dynabeads M-280 biotinylated

(50 µL; Invitrogen, USA) was aliquoted into 12 mL round bottom centrifuge tube and 8 mL of incomplete RPMI was added to wash the beads. The tube containing the beads was placed on a magnetic stand and the beads washed thrice as described before. Anti-rat IgG biotinylated (15 µL in 1 mL incomplete RPMI) was added to the beads and incubated (30 min; room temp) gently on a rocking table. The tube containing the mixture was placed on a magnet and the wash process repeated thrice to remove unbound antibodies. The bound PF11_0008, HB3 or IT4 parasites were added to the antibody-coated beads and incubated (30 min; 37 °C). All selected parasites were subsequently washed thrice as described before, resuspended in culture medium (7 mL) and 200 µL of human erythrocytes was added. The selected parasite suspensions were transferred to 25cm^2 culture flasks, gassed and maintained in an incubator (37 °C).

The beads were removed next day when parasites were at the ring stage. Briefly, the culture medium was aspirated and the parasites resuspended in fresh complete medium (10 mL). The parasite suspension was transferred to round bottom tubes, placed in a magnetic stand, inverted gently a few times and allowed to stand for 1 min. The medium containing the parasites were transferred to culture flasks, gassed and maintained at 37 °C. Giemsa-stained smears were prepared to assess parasitaemia and confirm parasite stage. The parasites are known to frequently alter their PfEMP1 expression and so the procedure was regularly performed every 2-3 weeks to maintain the predominantly expressed phenotype. The success of IE selection was routinely verified in transcription analysis using ring stage parasites and also flow cytometry assays by IE surface staining using the specific antisera or monoclonal antibody. Cultures with more than 60 % antibody-labeled IEs were used in the experiments.

3.3.14 IEs Surface reactivity to specific antibodies by flow cytometry

The phenotypic identity of the *P. falciparum* clonal isolates (3D7, HB3, IT4) was assessed using flow cytometry as described before (Joergensen *et al*., 2010). Briefly, late stage IEs were MACS purified and 100 µL (2×10^6 cells/mL) of IEs was added to wells containing specific rat antisera (1:40) for the various isolates (anti-PFD1235w_D4; anti-HB3VAR03_D4; anti-PF11_0008_D6; anti-IT4VAR13). The samples were incubated (30 min; 4 ^{0}C), washed twice by adding 200 µL of 2 % FBS and centrifuged (4 min; 500 x g; 4^{0}C). Thereafter, IEs were incubated (30 min; 4 ^{0}C) with FITC-conjugated goat anti-rat IgG (Vector Laboratories, USA) [1:150 in 2 % FBS; 100 µL/ well] and ethidium bromide (2 µg/mL; Sigma-Aldrich, Germany). The reaction mix was subsequently washed twice in 2 % FBS and the cells resuspended in 100 µL of 2 % FBS for acquisition of data on an FC500 MPL flow cytometer (Beckman Coulter, Denmark). Data were analysed using WinList version 6.0 (Verity Software House Inc.) and IEs were gated based on ethidium bromide positive cells.

3.3.15 Inhibition of IEs in flow-based adhesion assays

Flow adhesion and inhibition assays were performed on laboratory-adapted parasites (3D7, IT4, HB3) selected to express specific PfEMP1 using previously described methods (Lennartz *et al*., 2015). The assays were performed by Dr. Yvonne Adams (University of Copenhagen). Notably, 3D7 parasites were selected to express PFD1235w or PF11_0008, IT4-selected IEs expressed IT4VAR13 and selected HB3 IEs expressed HB3VAR03 (see 4.3.6). Channels of microslides (VI$^{0.1}$; Ibidi, Germany) were coated with recombinant ICAM-1-Fc [50 µg/mL], (Bengtsson *et al*., 2013a), EPCR-his [10 µg/mL] (Nunes-Silva *et al*., 2015) or with recombinant CD36 (rCD36; 20 µg/mL, R&D Systems, USA) and incubated in a humidified petri dish at

4°C overnight. Adhesion assays using human brain microvascular endothelial cells (HBMEC; Sciencell, USA) between passages two and five were seeded onto either fibronectin (2 μg/cm^2; Millipore, Germany) pre-coated venaEC (Cellix, Ireland) [5x10^5 cells/mL] or microslides (VI$^{0.4}$; Ibidi, Germany; 3x10^5 cells/mL) for 72–96 h. Stimulation of HBMEC was done by incubating with TNF-α (10 ng/mL; Thermofisher Scientific, USA) for 18 h. Microslides or venaEC (Cellix, Ireland) were blocked (1 h; 37 ^{o}C) with 1 % bovine serum albumin (BSA; Saveen Werner AB, Sweden) in PBS, mounted onto a Leica inverted phase contrast microscope and varying shear stresses (0.5 – 2 dyn/cm^2) were generated by connecting to an NE-1002X microfluidic pump (World Precision Instruments, UK). Cultured late-stage IEs adjusted to 3 % parasitaemia in RPM1 1640 with 2 % normal human serum (Copenhagen Hospital Biobank, Denmark) was subjected to a shear stress by passing or flowing over the microslides or venaEC for 5 min followed by another 5 min to wash off unbound IEs. The number of bound IEs per mm^2 for five separate fields was counted at 20x magnification. The flow was stopped and adherent IEs were removed after each run and the experiments repeated for the next shear stress. All experiments were performed in triplicates and repeated at least three independent times. Flow channels were pre-incubated with 40 μg/mL anti-ICAM-1 antibody (clone 15.2, AbD Serotec, USA), 10 μg/mL anti-EPCR (polyclonal, R&D Systems, USA) or with 20 μg/mL anti-CD36 (monoclonal, Beckman Coulter, Germany) to test the specificity of IE adhesion to ICAM-1-Fc, EPCR-his, rCD36 and HBMEC.

Inhibition of IE adhesion was carried out using the above protocol. Briefly, mature IEs were pre-incubated with affinity-purified IgG or human plasma (purified on D4 domain of 3D7 PFD1235w [PF3D7_0425800]) or motif antisera (targeting motif in 3D7 PFD1235w [PF3D7_0425800]), before being flowed on the microslides.

Negative controls included in the assay were affinity-purified non-immune rat or human IgG (Sigma-Aldrich, Germany).

3.3.16 Immunofluorescence assay

Equivalent molarity (4 mM) of ICAM-1-Fc and goat-F(ab)2-anti-human IgG-Fc Dylight 488 (Abcam, UK) or EPCR-His with mouse-anti-PENTA-His (Qiagen, UK) and anti-mouse IgG-Alexa 568 (Abcam, UK) were incubated (4 °C; 4 h), aliquoted and thereafter frozen (-20 °C) until ready for use. IEs (5 % parasitaemia) expressing PFD1235w, HB3VAR03, or IT4VAR13 were pelleted by centrifugation, washed with PBS and subsequently incubated with 2 % normal human serum (37 °C; 10 min). IEs were then washed twice with 1× PBS (Sigma-Aldrich, Germany) and incubated with thawed aliquots of the antibody: protein complex diluted 10x in 2 % Ig-free BSA (Sigma-Aldrich, Germany) diluted in PBS on a rocking table (37 °C; 10 min). IEs were washed twice with 2 % Ig-free BSA in PBS, air dried and mounted with ProLong Gold Antifade DAPI mounting medium (Life Technologies, USA) and examined using a Zeiss AxioImager Z1 equipped with a 63× oil inversion lens. Images were analysed with Zen Blue (Zeiss) and Fiji-ImageJ open source biological imaging software (Schindelin *et al*., 2012). Immunofluorescence assays were performed by Dr. Yvonne Adams (University of Copenhagen).

3.3.17 Bioinformatics analysis

Multiple alignments of DBLβ domains with binding affinity for ICAM-1 were generated using MUSCLE v. 3.7 software (Edgar, 2004) and the MEGA software

(Tamura *et al*., 2007) was used to create sequence distance trees. Bioinformatics analysis was done by Professor Anja Jensen (University of Copenhagen).

3.3.18 Data analysis

Clinical data of study participants were summarised as median, 25^{th} and 75^{th} percentile of values. Levels of *var* gene transcript among clinical groups were compared using the Wilcoxon-Mann-Whitney Rank Sum Test. To analyse whether study site influenced the association between ICAM-1 transcript levels and clinical outcome, a comparison was done using a logistic regression model with CM as outcome variable and study site (Benin, Ghana and Tanzania) as well as ICAM-1 transcript level as explanatory variables. Inhibition of ICAM-1 binding by antibodies was compared using the Wilcoxon-Mann-Whitney Rank Sum Test using SigmaPlot 13.0 (Systat Software Inc., United Kingdom). Adhesion inhibition experiments involving IEs were analysed by one-way ANOVA in GraphPad Prism 7.0 (GraphPad Software Inc., San Diego, USA) and IE binding to recombinant receptors or HBMEC was analysed by two-way ANOVA with Tukey's Multiple Comparison.

3.4 Results

3.4.1 Binding to ICAM-1 by DBLβ hybrid protein containing ICAM-1 binding motif

Bengtsson *et al*., (2013) showed that the C-terminal portion (4C region) of PFD1235w DBLβ3_D4 was responsible for ICAM-1 binding. In this study, the 4C region of DBLβ3_D4 was transferred to a related non-ICAM-1 binding DBLβ to generate a hybrid protein. Both the non-ICAM-1 binding DBLβ domain (DBLβ3_D5) and the

hybrid protein containing the minimal region involved in ICAM-1 binding (D5_motif) were tested using ELISA. The hybrid D5_motif protein bound to ICAM-1, whereas DBLβ3_D5 non-motif did not mediate binding to ICAM-1 (Figure 3.4A). Thus, the ICAM-1 binding property was gained by the non-ICAM-1 binding DBLβ protein.

3.4.2 Adhesion of IEs expressing motif-containing ICAM-1 binding DBLβ to ICAM-1

Further tests were carried out to determine whether antibodies raised against the ICAM-1 binding region would be capable of functionally inhibiting ICAM-1 binding to IEs. Briefly, rats were immunised with either the motif peptide or the DBLβ3_4 (D4) region mediating ICAM-1 binding. The resulting rat antisera were tested for their capacity to inhibit ICAM-1 binding of IEs expressing 3D7 PFD1235w, a group A PfEMP1 which is known to mediate ICAM-1 binding, a phenotype associated with cerebral IE sequestration (Bengtsson *et al*., 2013b; Turner *et al*., 1994). Purified anti-motif peptide IgG or DBLβ3_4 domain affinity purified on a 3D7 PFD1235w motif peptide were each incubated with selected mature IEs expressing PFD1235w, and thereafter allowed to adhere to recombinant ICAM-1 receptor under flow conditions. Controls in the assay included anti-ICAM-1 antibody, affinity purified non-immune rat IgG and rat antisera raised against IT4VAR13 which is a non DBLβ3_4 domain-containing ICAM-1 binder. Purified antibodies raised against DBLβ3_4 (α-D4) or the peptide (α-motif) significantly inhibited the binding of 3D7 PFD1235w IEs to ICAM-1 (p = 0.004, one-way ANOVA), whereas ICAM-1 binding by 3D7 PFD1235w IEs was not blocked by IT4VAR13 antibodies (Figure 3.4B). To test the functional significance of this inhibition, similar experiments were repeated with immune plasma affinity purified on both recombinant D4 and the motif peptide. Similar to the rat

antibodies, human IgG against the motif peptide and D4 showed a significant inhibition of the binding of IEs to ICAM-1 (p = 0.004; one way ANOVA; 3.4C). Thus, the data suggest that the motif sequence is sufficient to facilitate binding to ICAM-1.

3.4.3 Binding of ICAM-1 to recombinant DBLβ containing ICAM-1 binding motif predicted from sequence analysis

In order to determine the residues critical for ICAM-1 binding, structural analysis was done using a crystal structure of a group A PfEMP1 DBLβ domain in complex with ICAM-1 (performed by Professor Matthew Higgins' laboratory, Oxford University). Based on the data generated from the structural analysis together with the sequences in Bengtsson *et al*., 2013, a highly conserved sequence motif (I[V/L]x3N[E]GG[P/A] xYx27GPPx3H) harbouring the determinants of ICAM-1 binding by group A PfEMP1 was elucidated (Lennartz *et al*., 2017). The motif was used to search sequence databases resulting in the retrieval of 145 DBLβ domains with majority from group A PfEMP1 and a few B/A containing the motif and as such were predicted to mediate ICAM-1 binding (Lennartz *et al*., 2017). Based on these data, 26 randomly selected DBLβ domains were produced as recombinant proteins. ELISA was used to investigate ICAM-1 binding of 16 motif-containing DBLβ and 10 non-motif group A DBLβ domains (Appendix B). Among the 26 DBLβ proteins, eight were previously identified to bind ICAM-1 (Bengtsson *et al*., 2013b), but were identified as harbouring the motif in the sequence analysis (Lennartz *et al*., 2017). Ten proteins harbouring the motif and eight non-motif DBLβ proteins with unknown ICAM-1 binding properties were picked by sequence analysis of DBLβ sequences available from GenBank. The ELISA results showed that all of the 16 motif-containing DBLβ proteins bound

ICAM-1, although variability was observed in the degree of binding, whereas the 10 non-motif DBLβ were unable to bind (Figure 3.4D).

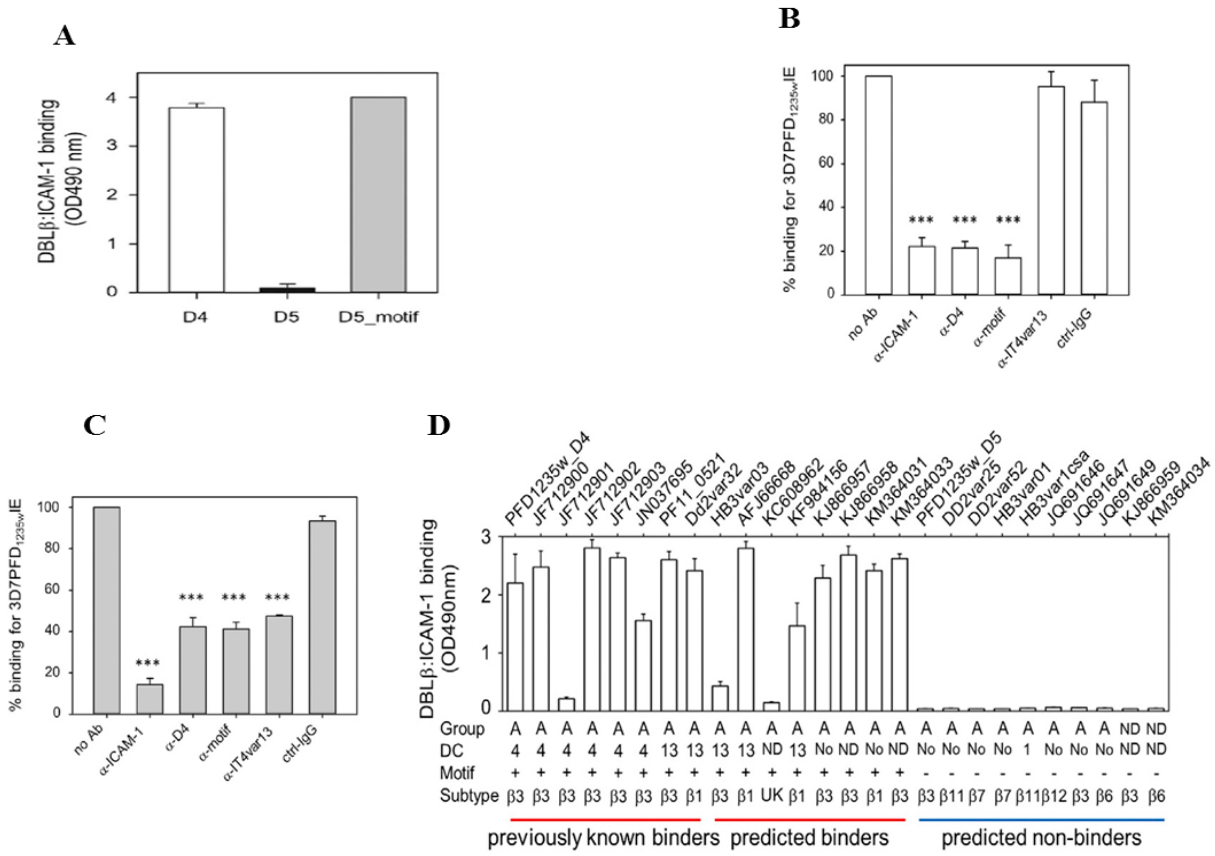


Figure 3.4: ICAM-1 binding of predicted DBLβ-containing ICAM-1 binding motif. (A). ICAM-1 binding by ELISA (OD 490 nm; ± SD) of recombinant 3D7 PFD1235w DBLβ3_D4 (motif-containing), PFD1235w DBLβ3_D5 (non-motif), and a hybrid of DBLβ3_D5 containing the ICAM-1 binding motif region of DBLβ3_D4 (D5_motif). **(B and C).** Inhibition of 3D7 PFD1235W IEs binding to ICAM-1 under flow conditions using anti-ICAM-1 antibodies, affinity-purified anti-PFD1235w DBLβ3_D4 (anti-D4) IgG, anti-PFD1235w_motif IgG, anti-ITVAR13 and control IgG. IgG antibodies were affinity purified from rat anti-serum **(B)** and human serum **(C)**. Results are shown as ±SD of a minimum of three independent experiments done in triplicate and data analysis was done using one-way ANOVA. * indicate significance ($p = 0.0004$). **(D).** ICAM-1 binding by ELISA (OD 490 nm; ± SD three replicates) of 26 recombinant group A DBLβ domains (4 DBLβ1, 14 DBLβ3, 2 DBLβ6, 2 DBLβ7, 2 DBLβ11, 1 DBLβ12 and 1 DBLβ unknown sub-class) from the structural analysis (Lennartz *et al*., 2017). All recombinant DBLβ-containing motifs bound ICAM-1, confirming the prediction of binding domains. ‘‘DC’’ stands for the domain cassette harbouring the domain. ‘‘ND’’ stands for unknown DC; the DC type is unknown as only the DBLβ sequence is available. ‘‘No’’ stands for a domain that is not part of a documented DC. Presence of DC13 prior to DBLβ is indicated.
Source: Lennartz *et al*., 2017

3.4.4 Phylogenetic analysis of ICAM-1 and non-ICAM-1 binding DBLβ domains

Phylogenetic analysis of DBLβ domains known to interact with ICAM-1 and DBLβ S3 sequences obtained from whole genome-wide studies revealed that group A and B/A PfEMP1 containing the ICAM-1 binding motif formed one phylogenetic group which differed from the other non-motif DBLβ in PfEMP1 of group A non-ICAM-1 binders, groups B and C ICAM-1 binders. Further analysis revealed that the PfEMP1-containing motif also contained an adjacent EPCR CIDRα domain. Indeed, they formed a separate phylogenetic cluster from non-motif containing PfEMP1 with EPCR binding domain and the motif shown to be present in about 14 % of all *var* genes encoding an EPCR binding CIDRα domain. Conversely, groups B and C PfEMP1 that mediate binding to ICAM-1 that are also linked to CD36 binding via CIDRα subdomain were found to be spread across the phylogenetic tree (Figure 3.5). Taken together, the data indicated the existence of at least two evolutionary divergent groups of ICAM-1 binding PfEMP1. Group A PfEMP1 with binding affinity for ICAM-1 formed a subgroup which was uniquely different from groups B and C PfEMP1 mediating ICAM-1 binding.

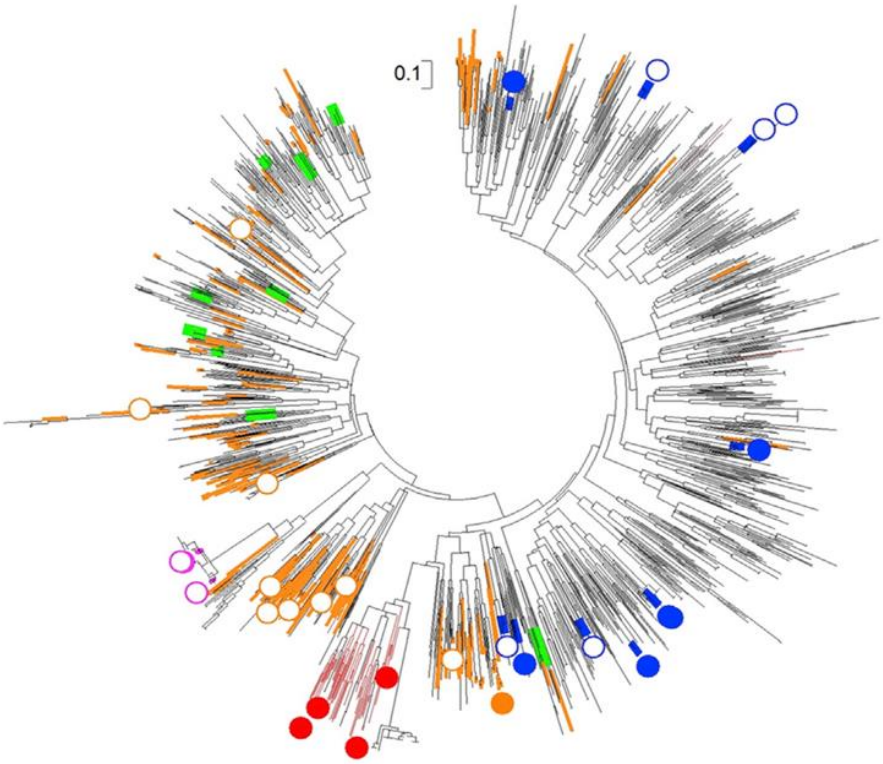


Figure 3.5: Phylogenetic tree of ICAM-1 binding and non-ICAM-1 binding DBLβ. The phylogenetic tree was constructed using 1,823 DBLβ S3 sequence data from the seven published genomes and 226 annotated whole genome sequencing data. Shaded and open circles stand for DBLβ experimentally demonstrated as ICAM-1 and non ICAM-1 binders respectively. Colors represent the CIDR domain located N-terminal to DBLβ: red stands for EPCR binders containing ICAM-1-binding motif; orange stands for EPCR binders without ICAM-1-binding motif; green stands for non-EPCR binders (group A); blue stands for CD36 binders; magenta stands for VAR1.
Source: Lennartz *et al*., 2017.

3.4.5 Cross-reactive adhesion inhibition by antibodies raised against motif-containing DBLβ motif-peptides

The sequence analysis data showed that the motif sequences were strikingly conserved among ICAM-1 binding group A DBLβ domains and that suggested that the motif might elicit serologically cross-reactive responses that may correlate with protection following natural *falciparum* infections. To test this, immune plasma samples from Liberian resident adults were pooled and purified separately on three different DBLβ domains containing the motif (Dd2VAR32, KM364031, and PFD1235w [PlasmoDB: PF3D7_0425800] DBLβ_D4) by affinity chromatography. Inhibition of ICAM-1

binding to different recombinant DBLβ proteins using the purified plasma IgG pool was assessed by ELISA. The pooled plasma IgG purified on the different motif-containing domains demonstrated the capacity to block ICAM-1 binding to a diverse selection of motif-containing DBLβ domains of group A PfEMP1 except for KF984156 (Figure 3.6A). For instance, the IgG purified on the Dd2VAR32 motif domain achieved greater than 74 % ICAM-1 inhibition in 10/13 motif-containing DBLβ domains; the IgG did not inhibit ICAM-1 binding of a group B DBLβ domain (Figure 3.6A). As a control, similar inhibitory experiments were carried out using the plasma pool affinity purified on a related non-ICAM-1 binding DBLβ domain without the motif (PFD1235w DBLβ _D5). The resulting IgG showed very little or no inhibition of ICAM-1 binding of the different recombinant DBLβ domains screened (Figure 3.6A).

To provide complementary data to support this cross-reactivity functionality, the ability of malaria-exposed children to acquire antibodies that could recognise the specific ICAM-1 binding site (motif peptide) present in the motif-harbouring DBLβ proteins and the extent to which these antibodies could interfere with ICAM-1 binding was determined. Briefly, a synthetic motif was used to screen plasma samples obtained from *falciparum*-exposed Tanzanian children. Majority of plasma samples (69/76; > 90 %) contained antibodies that had reactivity against the motif peptide. Further, plasma samples were affinity purified on the motif peptide and subsequently tested in ICAM-1 binding inhibition assays using DBLβ (D4 of PFD1235w). Notably, inhibition of ICAM-1 binding was markedly higher in plasma samples that demonstrated high motif peptide recognition (ELISA OD >1) relative to those with reduced reactivity towards the motif peptide (ELISA OD < 1; p=0.001) (Figure 3.6B). The data presented thus far showed that group A motif-containing ICAM-1 binding

PfEMP1 elicit cross-reactive IgG that prevents ICAM-1 adhesion to motif-containing DBLβ proteins.

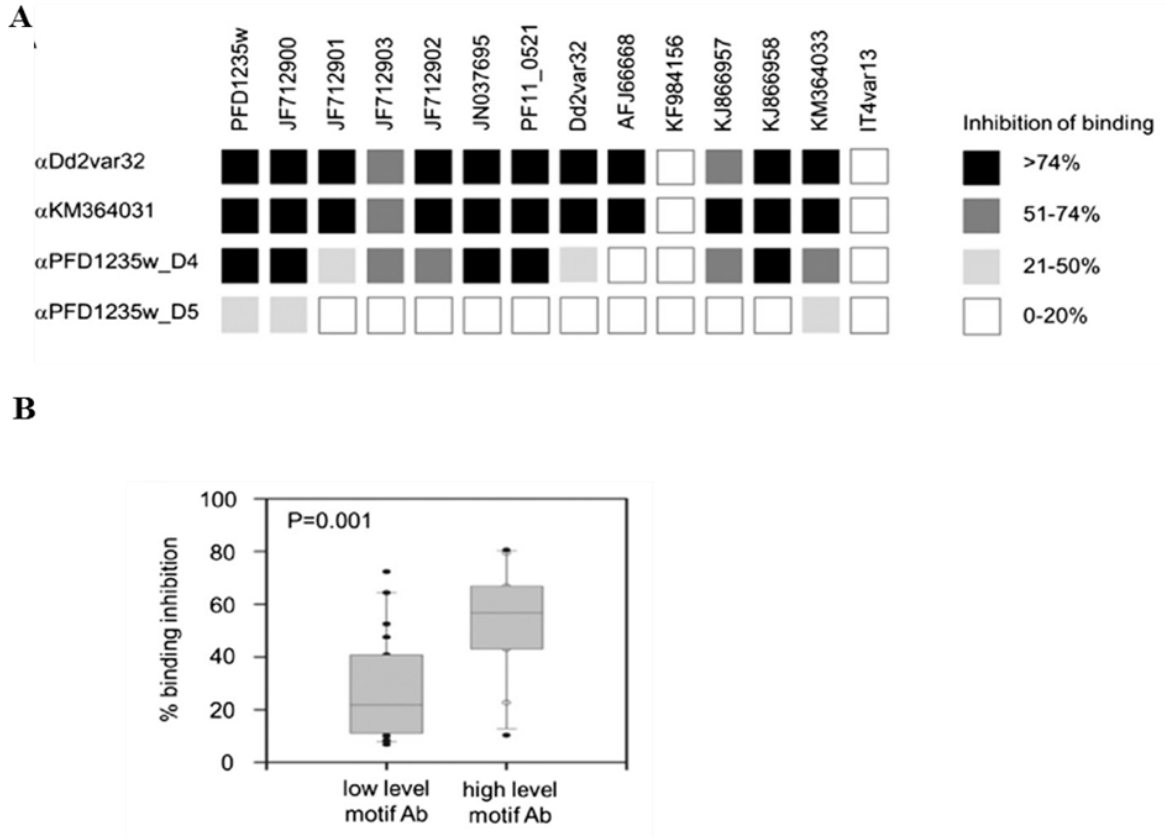


Figure 3.6: Binding inhibition of ICAM-1 to motif-containing PfEMP1 by plasma antibodies. (A). ICAM-1 binding inhibition by plasma pooled from adults. Purified IgG from plasma pool of immune adults inhibited ICAM-1binding of different motif-containing DBLβ domain variants (DBLβ_D4 domain from Dd2var32, KM364031, and PFD1235w) and no inhibition on a closely related, but non-ICAM-1-binding DBLβ_D5 domain from PFD1235w. ICAM-1 inhibitory capacity: >74% (black), 51%–74% (dark gray), 21%–50% (light gray), and 0%–20% (white). **(B)**. ICAM-1 binding inhibition by plasma antibodies from children. Plasma with low (ELISA OD < 1) and high (ELISA OD > 1) motif reactive DBLβ IgG in malaria-exposed Tanzanian children (1-17 years; Lusingu *et al*., 2004) inhibited ICAM-1binding to motif peptide. Results are shown as boxplots with median. Whiskers = 5% and 95% percentiles.
Source: Lennartz *et al*., 2017

3.4.6 Motif-encoding *var* transcript levels in SM patients

The transcription level of *var* genes encoding group A PfEMP1 was analysed to determine if there was a link to the different clinical malaria syndromes. In short, a specific primer set targeting the motif and in addition to primer sets specific for DNA sequences encoding EPCR binding CIDRα domains (highly transcribed in SM, Turner *et al*., 2013) were designed. Ring stage parasites obtained directly from 115 children with either CM or non-cerebral SM (Table 3.1) were purified and used to synthesize cDNA. qPCR was performed with the designed primer sets using the synthesized cDNA as templates. The relative transcription level of the *var* genes was determined by normalising to an endogenous housekeeping gene (*seryl-tRNA synthetase*) and further converted to transcript units, Tu (Lavstsen *et al*., 2012). High median transcript levels (Tu = 7.5) were recorded for motif-encoding DBLβ genes in CM in comparison with SA (Tu = 3.0) and other SM (Tu = 2.4). The median motif transcript values did not significantly differ between SA and other malaria; both groups were combined as one entity and subsequently compared with the CM group (Table 3.2). Median levels of transcripts encoding motif-containing DBLβ in CM group markedly differed from the combined non-cerebral SM group (p = 0.020). Analysis of *var* transcripts encoding CIDRα EPCR binders recorded a median level of Tu = 82.7 for CM, Tu = 46.4 for SA and median level of Tu = 77 was recorded for other malaria (Table 3.2). Thus, in comparison with motif transcripts, high transcripts of CIDRα EPCR binders were found among all the SM groups, consistent with previous findings that show high transcription of EPCR binding CIDRα domains in SM (Bertin *et al*., 2013; Turner *et al*., 2013; Lavstsen *et al*., 2012). A similar analysis of CIDRα encoding transcripts was done by combining the non-cerebral groups as one group and comparing with the CM group. The results showed no significant difference between

CM and the combined non-cerebral SM group (p=0.751). Taken together, the data suggest a connection between the expression of motif-encoding *var* genes and development of CM.

Table 3.1: Data on the study participants used for *P. falciparum var* gene expression experiments

Clinical characteristics		Gr. 1 CM	Gr. 2 SA	Gr. 3 OM
Country of origin	Benin	n= 34	n= 9	n= 16
	Ghana	n= 6	n= 2	n= 9
	Tanzania	n= 13	n= 15	n= 11
Age years		3.2 (2.5; 4.8)[a]	3.7 (2.1; 4.1)	2.5 (1.6; 3.6)
		5.0 (4.7; 5.3)	2.5 (2.0; 3.0)	4.8 (4.5; 5.6)
		2.3 (1.4; 4.0)	1.0 (0.4; 2.0)	2.8 (2.2; 3.8)
Blantyre coma score		2 (2; 2)	5 (5; 5)	5 (5; 5)
		1 (0; 2)	5 (5; 5)	5 (3; 5)
		1 (0; 2)	5 (5; 5)	5 (5; 5)
Haemoglobin (g/dl)		6.7 (4.8; 7.2)	4.3 (2.9; 4.3)	7.0 (5.8; 8.9)
		7.4 (7.0; 7.6)	4.8 (4.7; 4.9)	10.5 (6.6; 11.7)
		6.6 (6.1; 8.6)	4.2 (3.3; 4.6)	11.0 (10.7; 11.8)
Parasites per µl (x1000)		36.9 (9.3; 128.0)	40.7 (9.3; 116.0)	179.4 (23.0; 496.0)
		71.4 (10.7; 96.2)	56.4 (21.5; 91.3)	77.5 (13.7; 182.9)
		31.6 (3.3; 57.4)	50.4 (7.6; 86.5)	68.8 (20.1; 78.5)

Children admitted to the hospital with malaria were classified according to the WHO guidelines as CM: cerebral malaria: Blantyre coma score (BCS) $\leq$ 2 and no other identifiable course for the coma; SA: severe anaemia: haemoglobin <5g/dL and BCS >2; OM: other malaria, children admitted to hospital with BCS >2 and haemoglobin >5g/L. [a]Median (25 %; 75 % percentiles).
Source: Lennartz *et al*., 2017.

Table 3.2: *Var* subtypes transcript levels in children admitted with malaria

Predicted binding phenotype	Cerebral malaria (CM) *(n= 53)*	Severe anemia (SA) *(n= 27)*	Other malaria (OM) *(n= 36)*	CM vs. SA+OM, *P-value*
ICAM-1 binding Gr A DBLβ	7.5 [1.4; 32.9]	3.0 [1.0; 8.9]	2.4 [1; 12.3]	0.020
EPCR binding CIDRα	82.7 [36.4; 171.3]	46.4 [22.7; 126.2]	77.0 [29.9; 154.3]	0.751

Transcription levels of *var* genes measured using motif-specific primers, EPCR binding CIDRα primers and cDNA from ring stage parasites isolated from study participants. The relative transcription level was determined using an endogenous housekeeping gene (*seryl t-RNA synthetase*) and then converted to Tu (arbitrary units). Data are presented as median with 25 % and 75 % percentiles.
Source: Lennartz *et al*., 2017

3.4.7 Selection of parasites expressing PfEMP1 domains predicted to bind both EPCR and ICAM-1

Results from the bioinformatics and sequence analysis indicated that all PfEMP1 containing the motif additionally harboured an adjacent CIDRα domain known to mediate EPCR binding (Lennartz *et al*., 2017). This suggested that IEs expressing a combination of these domains to bind both ICAM-1 and EPCR. The study further hypothesized that PfEMP1 harbouring the motif and being able to bind EPCR are likely to be involved in CM pathogenesis.

To test this, HB3 and 3D7 IEs were antibody-selected (see section 3.3.13) to obtain IE expressing HB3VAR03 and PFD1235w, two PfEMP1 proteins known to mediate binding to ICAM-1 and EPCR. Additionally, IT4 IEs were antibody selected to enrich for IE expressing IT4VAR13, a dual CD36 and ICAM-1 binding PfEMP1. Finally, 3D7 IEs were selected to express PF11_0008 PfEMP1 (PlasmoDB: PF3D7_1100200), which is known to bind PECAM-1 (Joergensen *et al*., 2010). Surface expression of HB3VAR03, 3D7 PFD1235w, PF11_0008 and IT4VAR13 were confirmed by *var* transcription and flow cytometry and the data are shown in figure 3.7. *Var* transcription using gene-specific primer sets for the different parasite isolates showed the prominent transcription of PFD1235w in 3D7 IEs (Figure 3.7B), VAR03 in HB3 IEs (Figure 3.7D), and VAR13 in IT4 IEs (Figure 3.7F). In 3D7 PF11_0008 PF11 IEs (Figure 3.7H), in addition to the dominant expression of the selected *var* gene, minimal transcripts of other *var* genes were also observed. Results from the flow cytometry experiments in figure 3.7 (3.7A, 3.7C, 3.7E, 3.7G) showed that the different IE populations were homogenously labeled by the specific antisera and this was reflected by a shift in the fluorescence intensity of the positive population in comparison with unstained IEs.

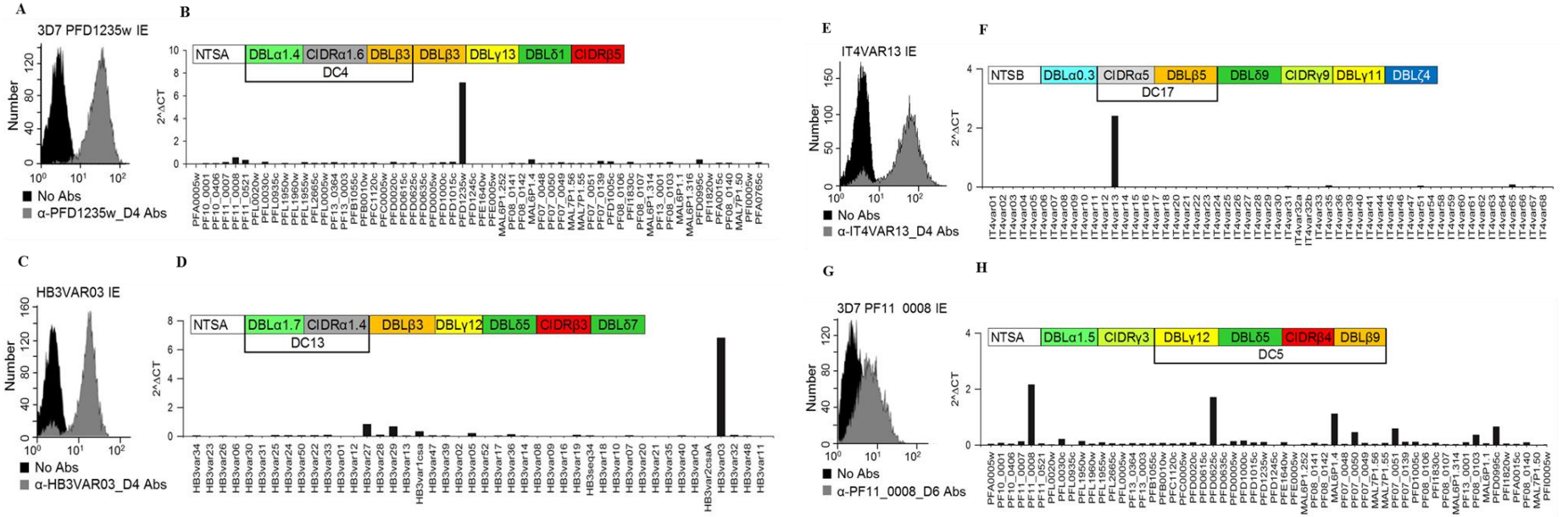


Figure 3.7: var/PfEMP1 expression of HB3, IT4 and 3D7 IEs. Specific primers for the different IEs were used in qPCR on cDNA isolated from ring stage-selected IEs. Transcription profile shows the dominant *var* transcripts following antibody selection. Flow cytometry data of different late stage-selected IEs stained with ethidium bromide and specific antisera. **(A)**. 3D7 PFD1235w ethidium bromide stained IEs with (grey histogram) and without (black histogram) rat PFD1235w DBLβ3_D4 antiserum. **(B)**. *Var* gene transcript levels present in 3D7 PFD1235W IE normalised with a housekeeping gene (*seryl-tRNA synthetase*). The domain organisation of the prominently expressed PfEMP1, PFD1235w is indicated. **(C)**. HB3VAR03 ethidium bromide stained IEs with (grey histogram) and without (black histogram) rat VAR03_D4 antiserum. **(D)**. *Var* gene transcript levels present in HB3VAR03 IE normalised with a house keeping gene (*seryl-tRNA synthetase*). The domain organisation of the prominently expressed PfEMP1, VAR03 is indicated. **(E).** IT4VAR13 ethidium bromide stained IEs with (grey histogram) and without (black histogram) rat VAR13_D4 antiserum. **(F)**. *Var* gene transcript levels present in IT4VAR13 IE normalised with a housekeeping gene (*seryl-tRNA synthetase*). The domain organisation of the prominently expressed PfEMP1, VAR13 is indicated. **(G)**. 3D7PF11_0008 ethidium bromide stained IEs with (grey histogram) and without (black histogram) rat PF11_0008_D6 antiserum.(H) *Var* gene transcript levels present in 3D7 PF11_0008 IE normalised with a house keeping gene (*seryl-tRNA synthetase*). The domain organisation of the prominently expressed PfEMP1, PF11_0008 is indicated.
Source: Lennartz *et al*., 2017

3.4.8 Adhesion of selected IEs to immobilised recombinant ICAM-1 and EPCR receptors

The capacity of selected IEs (3D7 PFD1235w, HB3VAR03, IT4VAR13) to adhere to recombinant receptors were analysed in a flow-based system using physiologically relevant shear stresses (0.5-5 dyn/cm^2). Recombinant human receptors ICAM-1, EPCR, CD36, or combinations of ICAM-1/EPCR or ICAM-1/CD36 were coated on channels of microslides and the selected IEs were passed on the pre-coated microslides for them to adhere. The binding assays were performed in triplicates, repeated at least three independent times and the average number of IEs/mm^2 per surface area estimated. Data are shown in figure 3.8. HB3VAR03 and PFD1235w IEs were observed to bind ICAM-1 and EPCR independently from 0.5-1.0 dyn/cm^2 with higher numbers bound at 1.0 dyn/cm^2 (Figures 3.8A; 3.8B). Binding to individual receptors markedly decreased at 2.0 dyn/cm^2 with nearly no detectable binding in HB3VAR03 IEs (Figure 3.8A). When both receptors were available at the same time, a high number of IEs were bound at 1.0 dyn/cm^2 compared with 0.5 dyn/cm^2 similar to results obtained for binding to the individual receptors. However, IEs were still bound at 2.0 dyn/cm^2, which is comparable to numbers observed at 1 dyn/cm^2 (Figure 3.8A; 3.8B). This observation at 2.0 dyn/cm^2 sharply contrasts the data generated from binding to individual receptors alone.

The dual ICAM-1 CD36 binding IEs (IT4VAR13) showed increased binding from 0.5-1.0 dyn/cm^2 to ICAM-1 receptor alone or when both receptors were simultaneously available (Figure 3.8C). Higher number of IT4VAR13 IEs adhered when both receptors were present in comparison with adhesion to CD36 receptor only. Binding to CD36 however, decreased after 0.5 dyn/cm^2 to almost no-detectable levels at 1.0 dyn/cm^2. At a high shear stress of 2.0 dyn/cm^2, binding to individual or

combined receptors sharply decreased (Figure 3.8C). Thus, the observed result was noticeably different from the binding observed for the EPCR and ICAM-1 dual binding parasites and it suggested that the former parasites (HB3VAR03 and PFD1235w IEs) are able to withstand binding at high shear stress. This data were further supported by immunofluorescence assays which showed binding of ICAM-1 and EPCR on the surfaces of both HB3VAR03 IEs and 3D7 PFD1235w IEs, whereas ICAM-1 was only localised on the surface of IT4VAR13 IEs (Figure 3.8E-3.8G). Control experiments were conducted with IEs selected to express PF11_0008 PfEMP1 (PlasmoDB: PF3D7_1100200) which is known to bind to PECAM-1 (Figure 3.8D). No binding was observed to any of the receptors investigated.

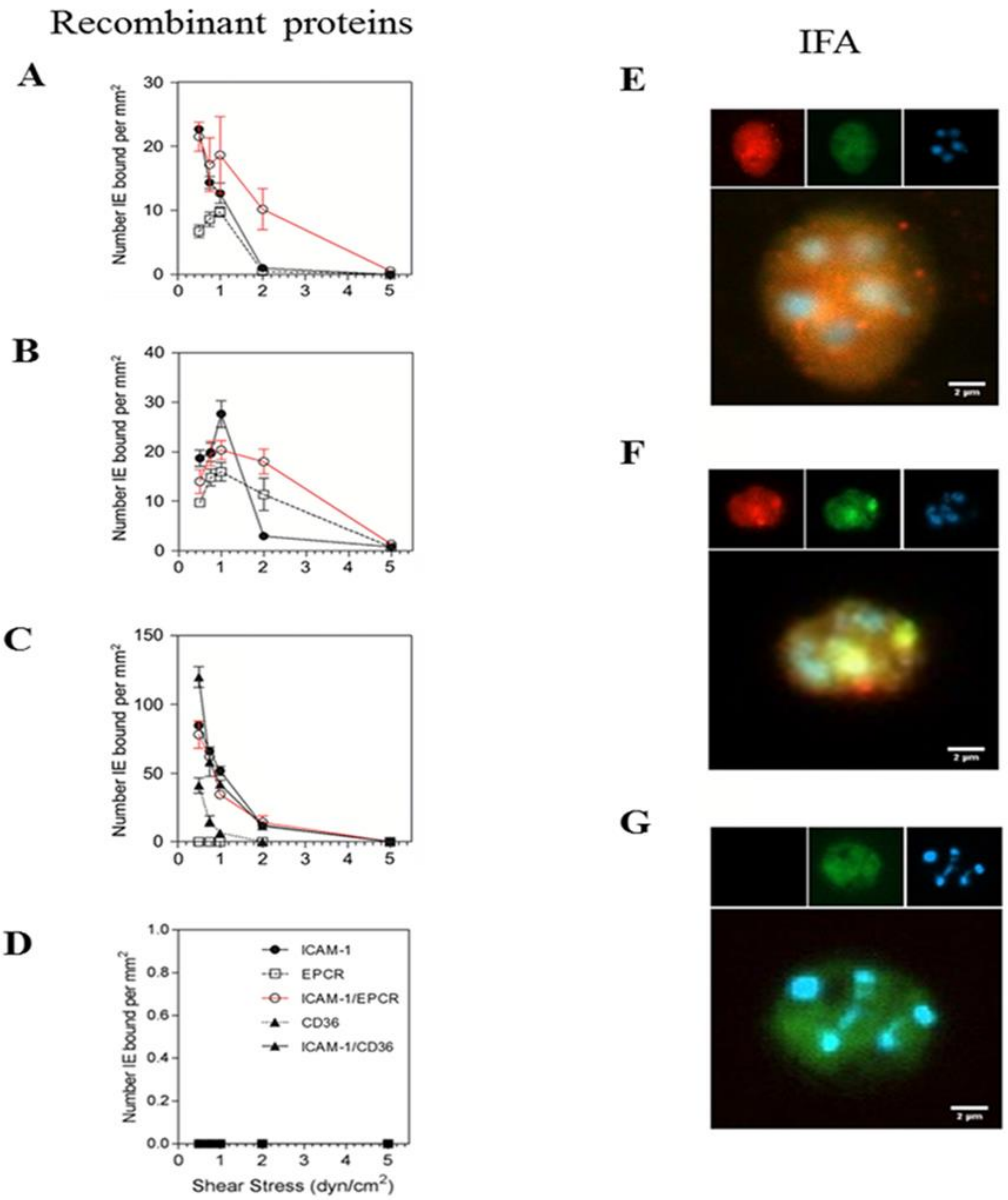


Figure 3.8: Binding of *P. falciparum* laboratory isolates to recombinant protein receptors. (A-D). Channels of microslides were pre-coated with rICAM-1, rEPCR, rICAM-1 and rEPCR; rCD36; or rICAM-1 and rCD36. Binding of **(A)** HB3VAR03 IEs (predicted ICAM-1 and EPCR binder), **(B)** 3D7PFD1235w IEs (predicted ICAM-1 and EPCR binder), **(C)** IT4VAR13 IEs (ICAM-1 and CD36 binder) and **(D)** 3D7PF11_0008 IE (PECAM-1 binder) to the immobilised receptors was performed in parallel at variable shear stresses (0.5 to 5.0 dyn/cm^2). Results are triplicates of at least three independent experiments and presented as bound IEs per mm^2 ± SEM (y-axis) plotted against shear stress (x-axis). Data were analysed by two-way ANOVA with Tukey's Multiple Comparison. **(E-G)**. Images from immunofluorescence assays showing IEs of **(E)** HB3VAR03, **(F)** 3D7PFD1235w, and **(G)** IT4VAR13. Complexes of ICAM-1or EPCR were incubated with the different IEs to assess binding by immunofluorescence. Results show overlays of ICAM-1 (green), EPCR (red), and nuclear (blue) staining. Inserts show single channels. Scale bar, 2 µm.
Source: Lennartz *et al*., 2017

The physiological consequence of the dual binding property of motif-containing PfEMP1 was investigated using HBMEC which is known to constitutively express ICAM-1 and EPCR on their surface. Binding was done using both resting and TNF-α stimulated HBMEC cells (passages 3-5). Blocking antibodies to each receptor were used to evaluate the influence of either ICAM-1 or EPCR binding alone or the effect of the combined receptors in binding. Shear stresses (0.5-2.0 dyn/cm^2) similar to the experiments done with recombinant proteins were used. The data are presented in figure 3.9. It was observed that HB3VAR03 IEs were bound to HBMEC that had not been pre-incubated with any receptor antibody between 0.5-2.0 dyn/cm^2 shear stresses (Figure 3.9A). By contrast, pretreatment with antibodies against either EPCR or ICAM-1 receptors slightly reduced the binding of HB3VAR03 IEs to HBMEC from 0.5-0.75 dyn/cm^2. Furthermore, a significant blockage in the binding of HB3VAR03 IEs to HBMEC was already observed at a low shear stress of 0.5 dyn/cm^2 in the presence of combined antibodies against EPCR and ICAM-1 (80 %, p = 0.0361). At increasing shear stress, a similar binding reduction pattern of HB3VAR03 IEs to HBMEC was further observed in the presence of either single receptor or combined receptor blockage. At a shear stress of 1.0 dyn/cm^2, a significant reduction in adhesion of HB3VAR03 IEs to HBMEC treated with individual receptor antibodies was recorded (Figure 3.9A).

Inhibition of 3D7 PFD1235w IEs binding to HBMEC blocked with either single receptor antibody or combined anti-EPCR and anti-ICAM-1 antibodies were observed at all the tested shear stresses for the various conditions (Figure 3.9B). This observation differed in comparison with the pattern of inhibition observed in HB3VAR03 IEs. Equivalent experiments were carried out with the dual ICAM-1 CD36 binding isolate IT4VAR13 IEs. HBMEC treated with anti-EPCR antibodies did

not affect the binding of IT4VAR13 IEs to ICAM-1 as expected, whereas HBMEC blocked with either ICAM-1 antibodies or the combined antibodies were inhibited at all shear stresses (Figure 3.9C).

Similar adhesion inhibition assays were done with HBMEC stimulated overnight with TNF-α using all the parasite lines. In the absence of any antibody treatment, increased binding to stimulated HBMEC by all IEs was observed (Figure 3.9D-3.9F) in comparison with binding on resting HBMEC at shear stresses between 0.5-2.0 dyn/cm^2. When stimulated and blocked with anti-EPCR antibodies, the number of HB3VAR03 IEs and 3D7 PFD1235w IEs bound were comparable to the numbers observed in stimulated cells only (Figures 3.9D, 3.9E). This may be due to the decreased EPCR expression following TNF-α treatment (Fukudome and Esmon, 1994). Thus, in the absence of EPCR, the parasites depended solely on ICAM-1 to attach to HBMEC. Treatment of HBMEC with either anti-ICAM-1 antibodies or combination of ICAM-1 and EPCR antibodies at all shear stresses blocked or prevented the binding of all IEs to stimulated HBMEC (Figure 3.8D-3.8F).

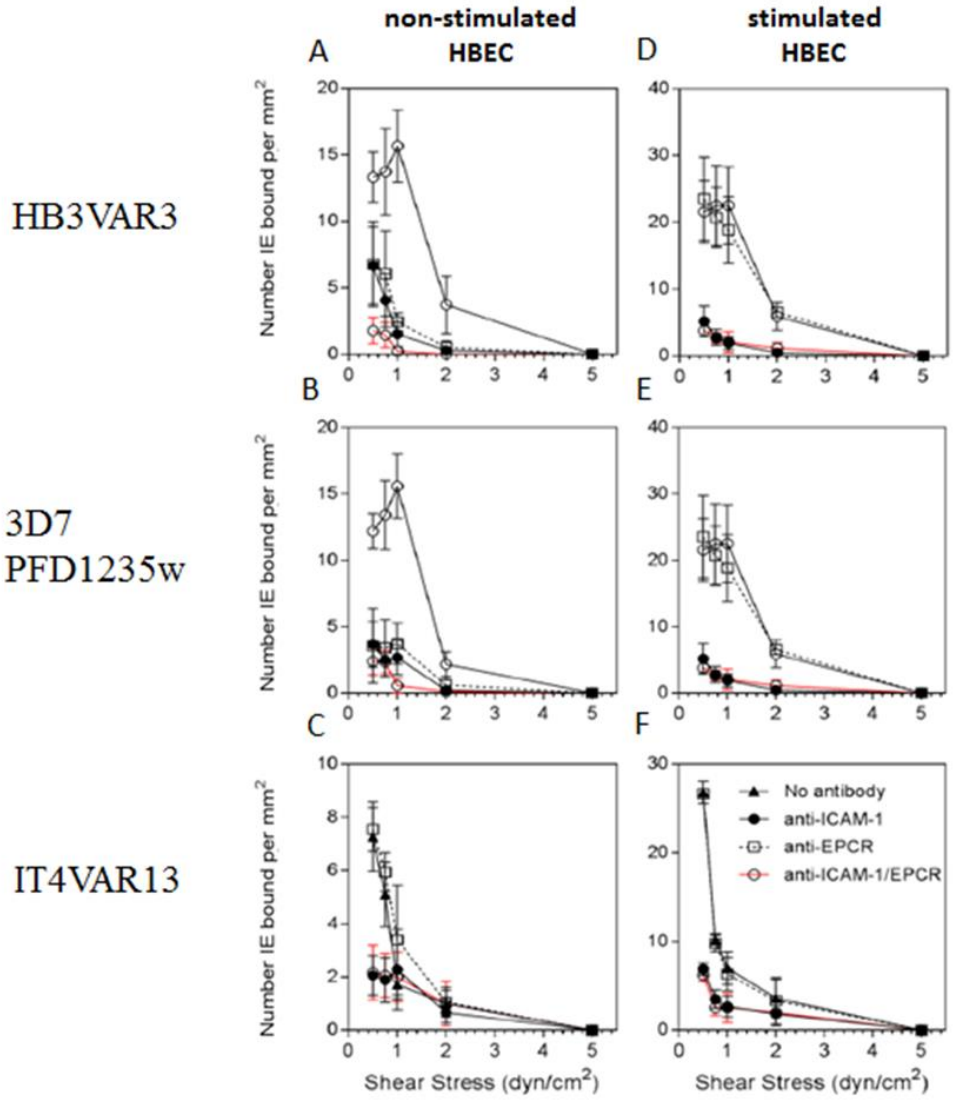


Figure 3.9: Binding of *P. falciparum* laboratory isolates to HBMEC under flow conditions. Binding of **(A and D)** HB3VAR03 IEs (predicted ICAM-1 and EPCR binder), **(B and E)** 3D7PFD1235w IEs (predicted ICAM-1 and EPCR binder), and **(C and F)** IT4VAR13 (ICAM-1 and CD36 binder) to resting **(A-C)** human brain microvascular endothelial cells (HBMEC) or TNF-α stimulated **(D-F)** HBMEC on pre-coated chips was performed in parallel at variable shear stresses (0.5 to 5.0 dyn/cm2). To assess specific adhesion, channels coated with HBMEC were pre-incubated with anti-ICAM-1 (40 µg/mL), anti-EPCR (10 µg/mL), or anti-ICAM-1 and EPCR combined (40 and 10 µg/mL, respectively). Results are triplicates of at least three independent experiments and presented as bound IEs per mm^2 ± SEM (y-axis) plotted against shear stress (x-axis). Data were analysed by two-way ANOVA with Tukey's Multiple Comparison.
Source: Lennartz *et al*., 2017

3.5 Discussion

The virulent parasite protein PfEMP1 is well recognised for its notorious sequence variability within and between the genome of different isolates. A mechanism adopted by the parasite to subvert the host immune system (reviewed in Chan *et al*., 2014; Miller *et al*., 2002). This has constituted a major bottleneck in considering PfEMP1 as an attractive vaccine immunogen. This significant impediment is highlighted in a study that used crystal structures of CIDRα1 bound to EPCR in an effort to identify conserved features that could be targeted for therapeutics against SM (Lau *et al*., 2015). They showed that the overall organisational structure of the CIDRα1 domain of PfEMP1 is preserved to mediate interaction with its receptor, however, the residues that directly interact with EPCR are significantly variable (Lau *et al*., 2015). Thus, remarkable sequence variability is observed even in PfEMP1 with the same binding phenotype and that significantly hampers the possibility of generating extensive cross-reactive natural responses against these CDIRα1 residues that directly interact with EPCR. Notwithstanding, this study used sequence information and structural analysis of a group A PfEMP1 protein bound to ICAM-1 to identify a highly conserved sequence motif. This motif was used to identify an array of DBLβ domains of group A PfEMP1 predicted to mediate ICAM-1 binding. Indeed, the recombinant DBLβ proteins containing an ICAM-1 binding motif predicted from the *in silico* analysis all successfully bound to ICAM-1 in contrast to the group A DBLβ lacking the motif. Furthermore, antibodies raised against the motif effectively blocked the interaction of IEs mediating ICAM-1 binding. Altogether, the data showed that the motif mediates ICAM-1 binding (Figure 3.4).

Previous studies have demonstrated that group A PfEMP1 are prominently expressed by parasites isolated from children with SM complications including CM and also

antibodies directed against group A PfEMP1 are acquired relatively faster and are believed to be cross-reactive (Bengtsson *et al*., 2013b; Cham *et al*., 2009; Joergensen *et al*., 2010; Jensen *et al*., 2004). This study provides evidence to support this notion and also demonstrates further that functionally important conserved epitopes indeed occur in group A PfEMP1 and are capable of eliciting cross-reactive responses. Such responses would be of great importance in combating CM development. qPCR data revealed markedly high transcription of motif-containing DBLβ in parasites obtained from CM in comparison with parasites from non-cerebral severe patients, showing a direct connection between the expression of motif-containing ICAM-1 DBLβ and development of CM (Table 3.2). Even more, data gathered from this study showed that individuals in malaria-endemic regions are naturally exposed to motif-containing DBLβ early in life; such individuals accumulated broadly cross-reactive functional IgG capable of inhibiting the interaction between ICAM-1 and motif-containing DBLβ domains of different *falciparum* genomes (Figure 3.6).

The identification of high levels of transcripts encoding motif-containing ICAM-1-binding DBLβ in CM provoked the question of why there have been inconsistent reports on the importance of ICAM-1 in CM development. While doing data mining of all currently available PfEMP1 sequences, it was discovered that all PfEMP1 that contained the motif additionally harboured an immediate upstream CIDRα domain and that potentially explained to some extent the discrepancy surrounding the connection between ICAM-1 and CM (Figure 3.5). Furthermore, it suggested why ICAM-1 could not have been the absolute receptor driving CM pathogenesis and also pointed to a likelihood of ICAM-1 and EPCR cooperation in CM. Previous studies have shown that IEs expressing PfEMP1 are capable of mediating adhesion to multiple receptors (Avril *et al*., 2016; Adams *et al*., 2014; Oleinikov *et al*., 2009;

McCormick *et al*., 1997). Recently, Avril *et al* (2016) showed that CIDRα mediating EPCR-binding and ICAM-1-binding DBLβ domains exist in a single PfEMP1. However, there were no documented reports on the ability of these PfEMP1 to engage both EPCR and ICAM-1 concurrently. Additionally, the benefits this dual adhesion offers the parasites were unknown. In this study, IEs expressing a single PfEMP1 capable of mediating adhesion to EPCR and ICAM-1 were selected (Figure 3.7) and assessed for binding under conditions that mimic *in vivo* blood flow, to understand the physiological impact of dual binding. IEs increased their ability to adhere at higher shear stress only when both EPCR and ICAM-1 were available for concurrent binding (Figures 3.8, 3.9). Upon TNF-α stimulation of cells, the IEs expressing the dual PfEMP1 domains markedly depended on ICAM-1 for binding, owing possibly to a decreased expression of EPCR (Fukudome and Esmon, 1994). Altogether, the results suggested that dual binding to EPCR and ICAM-1 equipped the parasite with flexible options that permitted enhanced cytoadhesion at physiologically higher shear stresses and also enabled the parasite to remain adherent during alterations in receptor expression resulting from cell stimulation. Thus, the process of increased cytoadhesion found in dual binding IEs facilitates the development of CM.

Stronger adhesion of IEs to both ICAM-1 and EPCR in the brain may drive disease pathogenesis through common pathways that involve both receptors and thus promote CM development. Indeed, adhesion of IEs to EPCR has been shown to disrupt the activation of protein C and further block downstream signaling pathways that regulate the vascular barrier properties that maintain the endothelium in an anticoagulant and anti-inflammatory state (Petersen *et al*., 2015; Turner *et al*., 2013). Even more, IE adhesion to EPCR results in a significant depletion of the normally low levels of EPCR on endothelial cells and an increase in adhesion molecules such as ICAM-1

(Moxon *et al*., 2013) that may promote further sequestration of IEs and increased pathology in the brain (Moxon *et al*., 2013). Thus, it is conceivable that the parasite is equipped with dual receptor binding capabilities to resist endothelial changes such as loss of EPCR functionality which occurs during IE binding or adhesion and still be retained in the brain by depending on an overexpressed ICAM-1 receptor for cytoadhesion.

Sequestration of IEs undoubtedly plays an important role in CM pathology (Ponsford *et al*., 2012; Silamut *et al*., 1999) and it is strongly associated with inflammation and more importantly contributes greatly to increased total parasite biomass, a critical determinant for development of CM (Storm and Craig, 2014; Cunnington *et al*., 2013a). The combined activities of sequestration and a perturbed inflammatory response give rise to endothelial dysfunction which may lead to alterations in the blood-brain barrier permeability, cerebral edema and coma (Sahu *et al*., 2015; Seydel *et al*., 2015; Storm and Craig, 2014; Idro *et al*., 2010). Brain swelling and increased intracranial pressure are notable features recorded in children with CM and it is a high risk factor for death (Seydel *et al*., 2015). Consistent with that, Seydel *et al* (2015) in a recent study reported severe brain swelling in 84 % of children who died from CM related complications in comparison with 27 % of survivours. Although the molecular details and sequence of CM pathogenesis remain partially elucidated, it is well recognised to be a complicated disease involving multiple factors including sequestration as well as other parasite and host factors that possibly converge to drive disease pathogenesis. Considering parasite proteins mediating sequestration, PfEMP1 appears not to be the only candidate involved. Emerging data show the possible involvement of other variant parasite proteins such as RIFIN and STEVOR proteins

(Goel *et al*., 2015; Niang *et al*., 2014). Thus, it is important for future studies to investigate the possible influence of all these aspects on the onset of CM pathogenesis.

In summary, this study provides evidence for the importance of an ICAM-1-binding motif in group A PfEMP1 with dual binding affinity for host proteins EPCR and ICAM-1. Such binding property allows for increased cytoadhesion and that constitutes an important risk factor for CM. The global burden associated with CM particularly in children remains unacceptably high even in those who recover. Thus, vaccines that will inhibit parasite sequestration in the brain and other supportive therapies that will aid full recovery from neurological deficits remain vital. Of interest, the residues characterising the motif are extremely conserved, thus, eliciting extensive cross-reactive responses and making them attractive candidates to be included in a vaccine cocktail that seeks to prevent CM complications.

CHAPTER FOUR

ANTIBODY RESPONSES TO PFEMP1 LINKED WITH THE DEVELOPMENT OF PAEDIATRIC CM

4.1 Introduction

SM syndromes in children, which include SA, respiratory distress and CM are thought to be driven by specific cytoadhesion properties of the parasite and an imbalanced host immune response (reviewed in Storm and Craig, 2014). Although massive disease intervention campaigns have been implemented for several decades, the burden of malaria remains unacceptably high particularly in children below the age of five in sub Saharan Africa (WHO, 2016). An effective vaccine to complement existing strategies remains a number one global health priority.

A striking immuno-epidemiological observation is that children from malaria endemic regions who survive few clinical episodes of malaria in the early stages of life become refractory to severe disease (Griffin *et al*., 2015; Goncalves *et al*., 2014; Reyburn *et al*., 2005; Gupta *et al*., 1999). Acquired immunity to febrile illness however, is remarkably slow and requires several years of persistent exposure to the malaria parasites. Moreover, lifelong exposure does not appear to offer sterile protection as adults in endemic regions are known to frequently harbour low levels of parasites, even though they are asymptomatic (reviewed in Fowkes *et al*., 2016; Bejon *et al*., 2014). Although the detailed mechanism of this natural acquisition is only partially understood, earlier studies have demonstrated that passive transfer of sera from immune adults to acutely ill children with malaria resulted in a profound reduction in disease severity (Sabchareon *et al*., 1991; McGregor *et al*., 1963; Cohen *et al*., 1961), providing strong evidence that antibodies targeting the asexual parasite stages are important. This antibody mediated clinical protection appears to be directed towards

the parasite variant antigens displayed on the surface of IEs particularly the PfEMP1 antigens (Chan *et al*., 2012; Ofori *et al*., 2002; Dodoo *et al*., 2001; Bull *et al*., 1998). PfEMP1 are encoded by the approximate 60 different members of the *var* multigene family and through a highly coordinated process only one or few PfEMP1 are expressed and exposed on the membrane of IEs (Hviid and Jensen, 2015). Periodically, the parasite alters the surface expression of these PfEMP1 variants to escape immune destruction (Scherf *et al*., 2008) and this likely translates into the reported slow rate of acquired clinical immunity.

In chapter three, a group A PfEMP1, harbouring an ICAM-1 binding motif, was identified via sequence analysis of several parasite genomes and parasites expressing this particular motif bind both ICAM-1 and EPCR concomitantly (Chapter three; Lennartz *et al*., 2017). This dual binding phenotype was associated with a high risk of developing CM and the PfEMP1 containing this motif among others included members of DC4 and DC13 (lennartz *et al* 2017). These findings suggest that antibodies directed against these motif-containing DCs could be central to acquisition of clinical immunity to CM. Therefore, this study aimed at investigating whether antibodies to the motif associated with CM are naturally-acquired in malaria-exposed populations and the possibility of experimentally inducing such motif-specific antibodies by immunisation.

4.2 Hypothesis and specific objectives

Motif-containing ICAM-1 DBLβ proteins induce cross–reactive antibodies that inhibit binding of IEs associated with CM to ICAM-1.

Specific objectives

1. To measure antibodies against motif-containing ICAM-1 binding and non-motif non-ICAM-1 binding DBLβ domains in plasma of malaria patients.
2. To generate motif-specific rat antibodies by immunisations and evaluate the reactivity and cross-adhesion inhibitory activities of these antibodies.

4.3 Methods

4.3.1 Study demographics

The study was carried out in the Hohoe Municipality (7º99N, 0°289E) which is located in the central zone of Ghana, approximately 220 km north east of Accra, with a population of 165,000 inhabitants. The study site was the Municipal Hospital located in Hohoe, with a population of 63,000 people (Kweku *et al*., 2015), has the responsibility of attending to referred cases from neighbouring towns. The climate in Hohoe Municipality is tropical (22 ^{0}C – 37 ^{0}C) and the vegetation is a semi-deciduous forest. Two rainy seasons are observed in a year and the major rainy season occurs from April to July each year. An average rainfall of approximately 1592 mm is recorded annually with more than 80 % of the rains occurring during the major rainy season. Malaria transmission is intense and all year round, with seasonal peaks observed after the major rains in June. The reported entomological inoculation rate is 65 infective bites per person per year (Kweku *et al*., 2015; Kweku *et al*., 2008). Malaria remains the principal cause of outpatient clinic visits (46.2 %) and constitutes about 30 % of total admissions. Among the top 10 causes of mortality, malaria and anaemia make up 16 % and 12 % respectively (Kweku *et al*., 2015). *Plasmodium*

falciparum, the dominant malaria parasite species in Hohoe remains sensitive to Artemisinin-based combination therapy (Quashie *et al*., 2013; Koram *et al*., 2005).

4.3.2 Patient samples and ethics

Children (< 12 years of age) admitted to the Hohoe Municipal Hospital with a microscopy diagnosis of *P. falciparum* malaria during the 2014 malaria transmission season were recruited after informed consent was obtained. Briefly, plasma samples (n=79) and *P. falciparum* isolates (n=13) were obtained from enrolled patients. Additional *P. falciparum* isolates (n=19) were obtained from a previous study (Lavstsen *et al*., 2012) on Tanzanian children diagnosed with malaria. Enrolles were categorised into SM or UM according to the WHO clinical and laboratory criteria (WHO, 2000). The criteria for SM included SA (Hb $\leq$ 5g/dL) and hyperparasitaemia (> 250,000 parasites/µL). CM patients (BCS $\leq$ 2) were not identified in this study. UM patients were identified by having parasitaemia less than 250,000 parasites/µL and Hb > 5 g/dL.

Plasma samples from 20 healthy Danes with no previous exposure to malaria were also included in this study. Both study sites received clearance from their respective ethics committee and a signed written informed consent from parents or legal guardians was obtained for each enrolled child (see chapter three section 3.3.1).

4.3.3 Recombinant proteins

Recombinant DBLβ domains containing the ICAM-1 motif or non-motif DBLβ proteins and a head structure of PFD1235w (CIDRα1.6- DBLβ3) were produced in *E. coli* SHuffle 3030 cells [New England Biolabs, UK] (Bengtsson *et al*., 2013b). Briefly, these proteins (Appendix C) were produced from genomic DNA amplified by

PCR using published primers (Bengtsson *et al*., 2013b), a new primer set (IT4VAR16 DBLβ_D4 primers, Appendix C) or produced as synthetic genes (DBLβ_D4 of CDO62031, CDO61797, and CDO63496) by Eurofins (Germany). The PCR products were subcloned into a modified pET15b vector and expressed as N-terminal, hexahistidine-tagged (His-tagged) proteins in *E. coli* SHuffle 3030 cells (New England Biolabs, UK). The expressed proteins were purified by immobilised metal ion affinity chromatography using HisTrap™ HP 1 mL column (GE Healthcare, Denmark). The motif peptide derived from PFD1235w DBLβ_D4 (LYAKARIVASNGGPGYYNTEVQKKDRSVYDFLYELHLQNGGKKGPPPATHP YKSVNTRDKRDATDDTTP) was produced by Schaefer-N, Denmark.

Recombinant GLURP-R2 (amino acids 705-1178) was produced in *E. coli* cells described previously (Theisen *et al*., 1995) and was kindly donated by Professor Michael Theisen. Human ICAM-1 combined with the Fc region of human IgG1 (ICAM-1-Fc) was cloned and expressed as a His-tagged protein in HEK293-F cells (Invitrogen, USA) as described previously (Bengtsson *et al*., 2013a). Recombinant ICAM-1 protein was purified from the supernatant on a HiTrap Protein G High-Performance column (GE Healthcare, Denmark), eluted using 0.2 M glycine/HCl buffer (pH 2.5), neutralized in suspension using Tris/ HCl buffer (1 M, pH 9) and finally buffer-exchanged into PBS (Bengtsson *et al*., 2013a).

4.3.4 DBLβ-specific antibodies and purification of motif-specific antibodies

All procedures concerning animal experiments and immunisations complied with the Danish national regulations. The animal procedures described in this study were carried out according to the guidelines elaborated in act numbers LBK 1306 (23/11/2007) and BEK 1273 (12/12/2005) and permission to carry out experiments

was granted by the Danish Animal Procedures Committee ("Dyreforsøgstilsynet") detailed in permit number 2008/561-1498. Before immunisation, sera were collected from each animal and used in flow cytometry and binding assays as negative controls. Recombinant PfEMP1 DBLβ constructs (3D7 PFD1235w_D4 [new ID: PF3D7_0425800_D4]; Dd2VAR32 [new ID: KOB85388] and KM364031_D4) and synthetic peptides were used to immunise rats to generate rat antisera. Procedures to those preparations have been previously described (Bengtsson *et al*., 2013b). Briefly, rats (Wistar; 8-12 weeks of age; three animals per immunisation group) were subcutaneously injected with 25 µg protein in complete Freund's adjuvant followed by two booster vaccinations of 15 µg in incomplete Freund's adjuvant. Post-immunisation sera were taken two weeks following the last immunisation.

Rat IgG specific for DBLβ proteins and motif (anti-PFD1235w_D4; anti-PFD1235w_motif; anti-IT4VAR13), were purified from rat antisera using previous procedures (Joergensen *et al*., 2010). Briefly, 1 mg of protein in each 1 mL was dialysed overnight against coupling buffer and further coupled to Hi-Trap NHS-activated High-Performance columns following the manufacturer's protocol (GE Healthcare, Denmark). Antibodies were diluted (1:1 in PBS), affinity purified, eluted in glycine buffer (0.1 M, pH 2.75) and neutralized in 1M Tris/HCl (pH 9.0).

4.3.5 ELISA

The anti-DBLβ reactivity was measured in plasma samples collected from the malaria patients. Maxisorp plates (Nunc, Thermofisher Scientific, Denmark) were coated with different recombinant DBLβ domains (50 µL; 2 µg/mL; 0.1 M glycine/HCl buffer pH 2.75) overnight at 4 ^{0}C. The plates were emptied and washed once with PBS-Triton-X-100 (PBS, 0.5 M NaCl, 1 % Triton X-100, pH 7.4) and blocked with blocking

buffer (PBS, 0.5 M NaCl, 1 % Triton-X-100, 1 % BSA, 0.03 mM phenol red, pH 7.2) for 30 – 60 min at room temp. The plasma samples were diluted 1:100 in blocking buffer, and 50 µL of prepared sample was added to each duplicate well and incubated (1 h; room temp). The plates were washed four times and bound antibodies in plasma were detected with an anti-human IgG-HRP (Dako, Denmark; 1:3000; in blocking buffer; 50 µL). Following 1 h of incubation and subsequent washing, plates were developed using O-phenylenediamine dihydrochloride tablets (Dako, Denmark) according to the manufacturer's instructions. The colour reactions were stopped by the addition of 50 µL of 0.5 M H_2SO_4, and the optical density (OD) values were read at 490 nm on a VERSAmax microplate reader (Molecular Devices, USA). Antibody reactivity was expressed as arbitrary units calculated as $(OD_{sample} - OD_{background})/(OD_{positive\ control\ sample} - OD_{background}) \times 100$ % (Jensen *et al.*, 2003). In all 26 recombinant proteins (25 DBLβ domains and 1 GLURP-R2) were used to screen 79 plasma samples and on each plate a positive hyperimmune African plasma pool and a negative non-exposed Danish pool were included.

Following the ELISA protocol above, antibodies raised in rats against motif-containing DBLβ domains of 3D7 PFD1235w (PF3D7_0425800), KM364031 and Dd2VAR32 (KOB85388) were tested for reactivity against the recombinant DBLβ constructs. Inhibition of ICAM-1 binding to DBLβ domains by these rat antisera was performed using a modified version of the ELISA protocol. Briefly, plates were coated with ICAM-1 (50 µL; 2 or 4 µg/mL ICAM-1; 0.1 M glycine/HCl buffer pH 2.75; overnight at 4 °C) and blocked with blocking buffer (1 h; room temp). His-tagged recombinant DBLβ domains (0.5-16 µg/mL) were mixed with antisera (1:5) or purified human IgG (10 µg/mL) and subsequently incubated (1 h; room temp) with pre-coated ICAM-1 plates. Following washing, bound DBLβ was detected with a

Penta-His antibody conjugated to HRP (Qiagen, UK; 1:3,000). All antisera were initially assessed for His-tag specific antibodies by ELISA and only sera negative for His-tag reactivity were included in the assay.

4.3.6 *Plasmodium falciparum* parasite culture

The *P. falciparum* clonal isolates (3D7, HB3, IT4) were cultured as described before (see chapter three section 3.3.9) and selected for IE surface expression of specific PfEMP1 (Bengtsson *et al*., 2013b; Joergensen *et al*., 2010). Briefly, 3D7 IEs were selected with the human monoclonal antibody AB01, which targets 3D7 PFD1235w DBLγ13_D6 [PF3D7_0425800_D6] (Barfod *et al*., 2011). *Plasmodium falciparum* IT4 IEs were selected using a rat antiserum against IT4VAR13 DBLβ5_D4 (ABM88750_D4), while the selection of HB3 IEs was done using a rat antiserum against HB3VAR03 DBLβ3_D4 (new ID: KOB63865_D4). The procedure was routinely done, the success of IE selection was verified in flow cytometry assay (see chapter three section 3.3.14) and only cultures with more than 60 % antibody-labelled IEs were used in the experiments.

A total of 33 *P. falciparum* parasite isolates obtained from Ghanaian and Tanzanian children were maintained in *in vitro* cultures for 1-28 days as described before (see chapter three section 3.3.9). Parasites were maintained in RPMI 1640 with 25 mM Hepes modified (Sigma-Aldrich, Germany), 50 µg/mL gentamicin sulfate (Sigma-Aldrich, Germany), 4 mM Glutamine (Sigma-Aldrich, Germany), 0.5 % Albumax (Invitrogen, USA), with 10 % pooled normal human serum (labelled as complete medium) and human erythrocytes (O+). The cultures were regularly checked for mycoplasma contamination using MycoAlert Mycoplasma Detection Kit (Lonza, USA) according to the manufacturer's protocol. The degree of parasite clonality was

determined by MSP 2 and GLURP genotyping using previously established methods (Snounou *et al*., 1999).

4.3.7 ICAM-1 Adhesion-Inhibition of IE under physiological flow conditions

Flow adhesion and inhibition assays were performed on patients' isolates and laboratory adapted parasites using previously described methods (Lennartz *et al*., 2015) and was performed by Dr. Yvonne Adams (University of Copenhagen). Microslides ($VI^{0.1}$; Ibidi, Germany) were coated with recombinant ICAM-1-Fc (50 µg/mL, Bengtsson *et al*., 2013a), at 4 ^{o}C overnight. Microslides were blocked (1 h; 37 ^{o}C) with PBS + 1 % BSA, mounted onto a Leica inverted phase contrast microscope and a shear stress (1 dyn/cm^2) was generated by connecting microslides to an NE-1002X microfluidic pump (World Precision Instruments, UK). Cultured late stage IEs adjusted to 3 % parasitaemia were subjected to a shear stress by flowing over the microslides for 5 min followed by another 5 min to wash off unbound IEs. The number of bound IEs per mm^2 for five separate fields was counted at 20x magnification and the experiment was performed in triplicates and repeated at least three more times independently.

Inhibition of ICAM-1 binding was carried out using *P. falciparum* isolates (3D7, HB3, IT4) following the above described protocol. Briefly, mature IEs were incubated with affinity-purified IgG (purified on D4 domains of 3D7 PFD1235w [PF3D7_0425800]) or motif antisera (targeting motif in 3D7 PFD1235w [PF3D7_0425800], Dd2VAR32 [KOB85388] or KM364031) before being allowed to flow on the microslides. Controls included in the assay were affinity-purified non-immune rat IgG and antisera against IT4VAR13 (new ID: ABM88750). ICAM-1 specific binding to IEs was tested by pre-incubating microslide channels with the anti-

ICAM-1 antibody (40 µg/mL, clone 15.2, AbD Serotec, USA). Inhibition assay on *P. falciparum* field isolates was tested on isolates that recorded ≥ 10 IE/mm^2 bound ICAM-1 in the adhesion assay. The assay was done using a pool of rat antisera (1:100 dilution) raised against motif derived peptides in 3D7 PFD1235w [PF3D7_0425800] and Dd2VAR32 [KOB85388].

4.3.8 Immunofluorescence assay

Immunofluorescence assays with mature IEs expressing PFD1235w, HB3VAR03, or IT4VAR13 were performed following previously described procedures (Ghumra *et al*., 2008) and was done by Dr. Yvonne Adams (University of Copenhagen). IEs (50 µL, 5 % parasitaemia) were centrifuged, washed twice with PBS with 1 % Ig-free BSA (Sigma-Aldrich, Germany) and incubated with sera (1:50; 1 h) on ice. Cells were washed thrice (PBS with 1 % Ig-free BSA) and incubated with anti-rat-FITC secondary antibody (Vector laboratories, USA) on ice for 1 h in the dark. Following washing, thin slides were prepared, parasite nuclei were stained with 5 µL ProLong Gold anti-fade DAPI (Thermofisher Scientific, USA) and the thin slides covered with slips. Images were obtained with a Nikon Eclipse TE2000 microscope and x63 objective.

4.3.9 Data analysis.

Patient data were presented as median and interquartile range (25th and 75th quartiles) except for male frequency which was summarised as percentages. Plasma antibody reactivity was expressed as ELISA units (EU, percentage) and a score was assigned for each plasma sample/protein combination in relation to the calculated EU. Briefly, EU between 0-25 % of the combination was assigned a score of 0; 26-50 % were

assigned a score of 1; 51-75 % were scored as 2; 76-100 % were scored as 3; above 100 % were scored as 4. The sum of all scores was calculated for each DBLβ recombinant protein and each plasma sample. Data were analysed by Student's unpaired t-test using the SigmaPlot 13.0 software (Systat Software Inc., United Kingdom). Statistical significance was set at $p \leq 0.01$. Experiments involving ICAM-1 adhesion-inhibition of laboratory-adapted IEs and IEs from patients were analysed by One-way ANOVA in GraphPad Prism 7.0 (GraphPad Software Inc., San Diego, USA). Multiple alignments of DBLβ domains with binding affinity for ICAM-1 were generated using MUSCLE v. 3.7 software (Edgar, 2004) and the MEGA software (Tamura *et al*., 2007) was used to create sequence distance trees.

4.4 Results

4.4.1 Characteristics of patients for the antibody reactivity profile

This hospital based study enrolled a total of 79 Ghanaian patients (<12 years of age). The characteristics of the qualified participants are shown in Table 4.1. SM was more frequently recorded in males whilst there was no gender bias in the occurrence of UM. Overall, the median age between SM patients (3.8 ± 0.3) significantly differed from those of UM (5.6 ± 0.4) (Student's unpaired t-test, $p = 0.0020$).

Table 4.1: General characteristics of patients related to figure 4.1

	Severe malaria		Uncomplicated malaria	
Age distribution	1-4	>4	1-4	>4
	(n=25)	(n=10)	(n=23)	(n=21)
Male gender (percentage)	64 %	70 %	43.48 %	52.38 %
Age in years	2.74*	6.65*	3.37	7.93
(25 %; 75 %)	(2.09; 3.27)	(5.42; 7.72)	(2.68;4.22)	(5.44;9.65)
Blantyre coma score	5.0	5.0	5.0	5.0
(25 %; 75 %)	(5.0;5.0)	(5.0;5.0)	(5.0;5.0)	(5.0;5.0)
Haemoglobin (Hb, g/dL)	8.7	10.3	9.6	10.9
(25 %; 75 %)	(5.15; 10.55)	(8.83;11.50)	(7.7;10.5)	(9.8;11.7)
Parasites (per µL*1000)	74.5	123.19	18.7	18.5
(25 %; 75 %)	(10.0;189.5)	(37.9;165.7)	(3.6;71.7)	(1.7;90.9)

Data are shown as median (1st and 3rd interquartile values) except male gender which is shown as percentages. Patients were grouped into SM (35) and UM (44). Within each disease severity group, patients were distributed into age categories (1-4 years, >4 years). Overall, SM was most frequent in males. Also, the median age was noticeably lower in patients with SM compared to patients with uncomplicated malaria. * Student's unpaired t-test between SM and UM, $p<0.01$.

4.4.2 Plasma antibody responses to DBLβ domains of group A PfEMP1

In chapter three, DBLβ domains of group A PfEMP1 with a conserved motif that bind ICAM-1 were found to be connected with the risk of CM development. Hence, this study sought to profile antibody responses against a large set of recombinant DBLβ constructs. A total of 25 different recombinant DBLβ domains which included 12 with motif-binding ICAM-1 and 13 non-motif non-ICAM-1 binding proteins derived from different *P. falciparum* parasite genomes were used to examine the antibody reactivity in plasma samples of 79 children by ELISA. The assay also included the C-terminal repeat region (R2) of GLURP protein, an asexual stage non PfEMP1 protein considered a vaccine candidate (Dodoo *et al*., 2000). Each ELISA plate contained a positive hyper-immune African control to normalise data. Plasma antibody reactivity was expressed as ELISA units (EU) and a score was assigned for each plasma sample/protein combination in relation to the calculated EU (see section 4.3.9). The mean sum of all scores was further calculated for each DBLβ recombinant protein and each plasma sample and was used to compare responses between motif-containing and non-motif DBLβ. Figure 4.1A shows reactivity profiles categorised into two domain subsets namely motif-containing (left panel) and non-motif DBLβ with both plotted against the age of the patient donors. In general, the pattern of antibody recognition to the different DBLβ domains greatly varied among the children (Figure 4.1A). Notably, low antibody levels were recorded against all screened DBLβ proteins for some donors (e.g. donors 2, 14, 16; Figure 4.1A), conversely a broad spectrum of DBLβ specific antibody responses with high titres were measured in plasma samples from other children (e.g. donors 50, 67, 70; Figure 4.1A). Comparative plasma antibody responses between the two domain subsets revealed that significantly lower mean score of antibody titres against motif-containing ICAM-1 binding DBLβ were

recorded (Figure 4.1B left panel) relative to antibody titres against the non-motif DBLβ proteins (Figure 4.1B right panel) (Student's unpaired t-test, $p \leq 0.001$). Thus, plasma samples from most children were markedly more reactive towards non-motif DBLβ than towards motif-containing ICAM-1 binding DBLβ proteins (Student's unpaired t-test, $p \leq 0.001$).

4.4.3 Antibody responses determined by age and disease severity

Antibody responses within the two DBLβ subsets (with or without the ICAM-1 motif) were stratified by age (1-4 years, > 4 years) and disease severity (UM, SM) and shown in figure 4.1C. Antibody reactivity compared among the age groups indicated an age-dependent accumulation in both motif containing (Figure 4.1C, left panel) and non-motif DBLβ groups (Figure 4.1C, right panel). Notably, children in the 1-4 years group recorded strikingly lower titres in comparison to children more than 4 years old (Student's unpaired t-test, $p \leq 0.001$). Within the younger age group (1-4 years), motif-containing specific antibody response outcomes remained markedly lower in comparison with responses against non-motif DBLβ proteins (Student's unpaired t-test, $p \leq 0.001$). By contrast, there was no significant difference in antibody reativity to both motif and non-motif proteins in the comparatively older age group (> 4years old). Further, comparable mean sum of scores for antibody responses were recorded for both SM and UM in motif-containing ICAM-1 binding DBLβ within the same age groups. An equivalent pattern was observed between disease severity groups of the non-motif DBLβ binders.

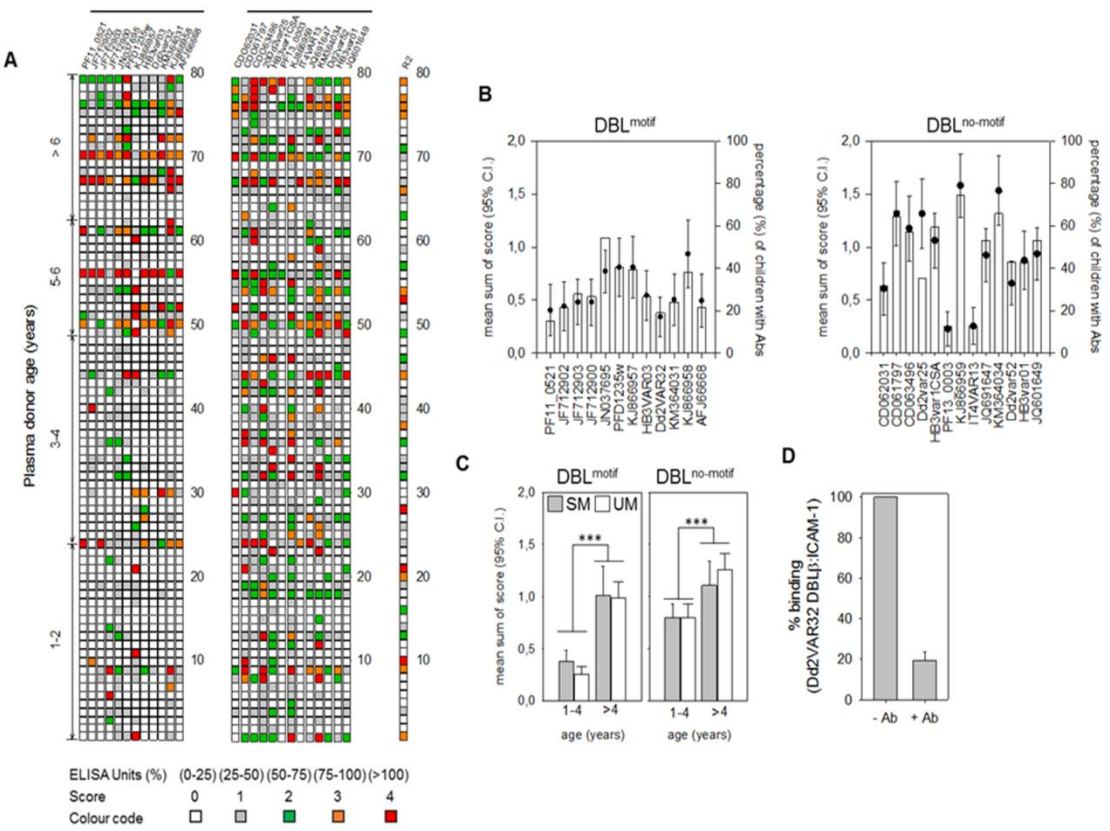


Figure 4.1: Plasma antibody reactivity to *P. falciparum* DBLβ domains. Plasma samples obtained from 79 pediatric patients with either severe or uncomplicated *P. falciparum* infections were tested for their reactivity against a broad selection of DBLβ domains and the responses were expressed as ELISA units (EU). **(A).** Antibody responses categorised into DBLβ domains with motif (left column) or non-motif (centre column); a non-DBLβ domain (negative control), R2 (right column). The antibody responses are plotted against age (left edge). The numbers along the right edge indicate the total number of plasma samples analysed (1-79). Each square stands for a specific DBLβ/plasma combination and colours indicate reactivity (%) relative to a positive control pool as indicated in the figure key and detailed in 4.3.5. **(B-C)**. Both figures similar to **(A)** show plasma antibody reactivity to each DBLβ domain; the responses were scored on a scale of 0 to 4 relative to the IgG reactivity to that particular domain. **(B)**. The overall antibody reactivity to each DBLβ domain was calculated as the mean sum of the score (bar charts) with 95 % confidence intervals (C.I.; error bars) from all 79 plasma samples and shown for motif-containing (left panel) and non-motif (right panel) DBLβ domains. The white bars in each panel represent the percentage of children with a positive antibody response. **(C)**. Antibody responses relative to age and disease severity are shown for motif-containing ICAM-1 binding DBLβ domains (left panel) and non-motif DBLβ domains (right panel). Calculation of the overall antibody recognition for all DBLβ domains was similar to those performed in **(B)**. **(D)**. A plasma pool was generated from the donors and used to determine the ability to inhibit ICAM-1 binding by DBLβ_D4 of Dd2VAR32 [KOB85388]. Gray bars represent percentage binding by DBLβ_D4 of Dd2VAR32 to ICAM-1 in the absence (left) or presence of (right) pooled plasma antibody. ***Student's t-test, $p \leq 0.001$.

4.4.4 ICAM-1 binding-inhibition by antibodies against motif-containing DBLβ domains

Plasma samples randomly obtained from ten children based on reactivity to motif DBLβ protein were pooled and used to test for the ability to inhibit ICAM-1 binding in an ELISA. The samples were pooled to increase the signal in the inhibition of binding assay. Briefly, the pooled plasma was mixed with a recombinant motif-containing DBLβ protein and subsequently incubated with recombinant ICAM-1 on pre-coated plates. The data presented in figure 4.1D showed that the pooled plasma contained antibodies that could markedly ($p < 0.001$) inhibit (> 80 %) ICAM-1 binding of a heterologous motif-containing DBLβ (Dd2VAR32_D4 [KOB85388_D4]) protein. This result suggested indirectly that the motif-containing DBLβ could induce cross-reactive antibodies that inhibit ICAM-1 binding. In order to investigate this hypothesis, antisera were generated from rats immunised with four recombinant motif-containing DBLβ constructs (DBLβ_D4 domains of 3D7 PFD1235w [PF3D7_0425800], PF11_0521 [PFD3D7_1150400], KM364031 or Dd2VAR32 [KOB85388]) identified as domains involved in ICAM-1 binding in chapter three. Similar inhibitory ELISA experiments as described above were carried out using rat antisera against these motif DBLβ domains (14 motif-binding ICAM-1 DBLβ and two non-motif ICAM-1 binding DBLβ). The results showed that the individual antisera had the capacity to interfere with ICAM-1 binding of homologous and several heterologous motif-containing DBLβ domains (Figure 4.2A). Conversely, these rat antisera showed minimal binding–inhibition of non-motif proteins of DBLβ_D4 domains of IT4VAR13 [ABM88750] (~ 30 %) and ITVAR16 [new ID AAS89259] (~ 40 %) [Figure 4.2A]. Experiments using pooled antisera against motif-containing

DBLβ resulted in pronounced inhibition (> 75 %) of ICAM-1 binding of all motif domains (Figure 4.2A).

The cross-inhibitory function of the specific IgG against the motif (the ICAM-1 interaction site) present in the DBLβ domain was determined. Briefly, serum IgG collected from rats immunised with any of the three motif-containing DBLβ domains (D4 of 3D7 PFD1235w; KM364031; or Dd2VAR32) were affinity purified on a peptide of the 3D7 PFD1235w motif and was tested in similar ELISA experiments as described before for the antisera against the motif DBLβ domains. Purified IgG against the motif exhibited the capacity to block ICAM-1 binding of nearly all except two DBLβ motif proteins (KM364031; KF984156). Conversely, purified motif IgG had no effect on the level of binding of the non-motif constructs DBLβ_D4 of IT4VAR13 and of IT4VAR16 (Figure 4.2B).

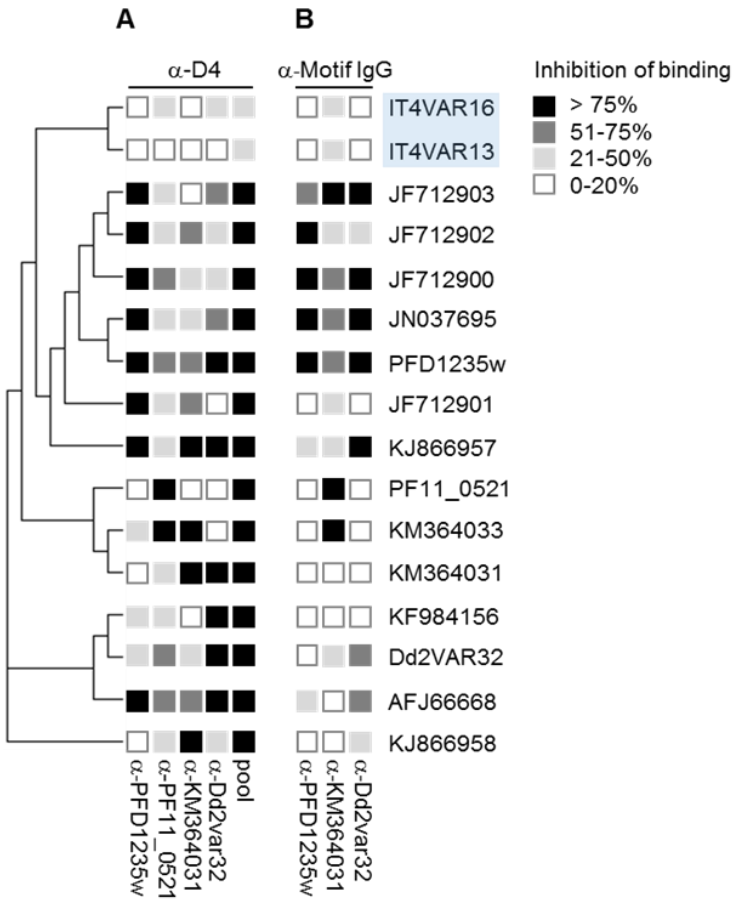


Figure 4.2: Inhibition of ICAM-1 binding by antibodies raised against motif-containing DBLβ domains. (A). Individual or pooled antisera generated from rat immunisations with 3D7 PFD1235w_D4 [new ID PF3D7_0425800], PF11_0521_D4 [new ID PFD3D7_1150400], KM364031_D4, and Dd2VAR32_D4 [new ID KOB85388] were used to test for the inhibition of DBLβ: ICAM-1 binding in ELISA. A phylogenetic tree indicating the relatedness of the different domains is shown to the extreme left. **(B)**. Similar inhibitory experiments carried out with 3D7 motif-specific IgG affinity purified from rats immunised with DBLβ_D4 of PFD1235w, KM364031 or Dd2var32. IT4VAR16 and IT4VAR13 indicate non-motif containing DBLβ. Level of inhibition is shown as: black (>75%), dark gray (50-75%), gray (20-50%), and white (<20%).

As described in chapter three, an important finding was that IEs selected to express motif-containing DBLβ domain (HB3VAR03, 3D7 PFD1235w) on their surface had dual binding affinity for human receptors EPCR and ICAM-1. The ability of motif-specific IgG to broadly inhibit the adhesion of ICAM-1 using laboratory adapted isolates (HB3, IT4 and 3D7) was therefore evaluated in binding assays under physiological flow conditions. Briefly, antisera raised against the motif-harbouring

proteins [3D7 (PFD1235w) and Dd2VAR32 (KM364031)] and IgG purified on the PFD1235w motif peptide were utilised. Both affinity purified motif IgG antibodies were individually incubated with selected mature IEs expressing PFD1235w, HB3VAR03 or IT4VAR13 and thereafter allowed to bind recombinant ICAM-1 receptor under a physiologically relevant shear stress of 1.0 dyn/cm^2. Affinity purified non-immune rat IgG and antisera against IT4VAR13 were included as controls. The results showed that both IgG purified on the motif peptide significantly inhibited the adhesion of HB3VAR03 ($p < 0.0001$) and 3D7 PFD1235w IE ($p = 0.0001$) to recombinant ICAM-1 (Figure 4.3). Specific IgG targeting the Dd2VAR32 motif were also observed to interfere with the binding of all three parasite isolates. Notably, antibodies targeting IT4VAR13 or non-immune control IgG did not interfere with ICAM-1 binding of HB3VAR03 nor 3D7 PFD1235w IE (Figure 4.3). Taken together, the data presented thus far showed that motif-containing DBLβ elicit cross-reactive inhibitory antibodies that block the interaction between IEs expressing surface specific native PfEMP1 and ICAM-1.

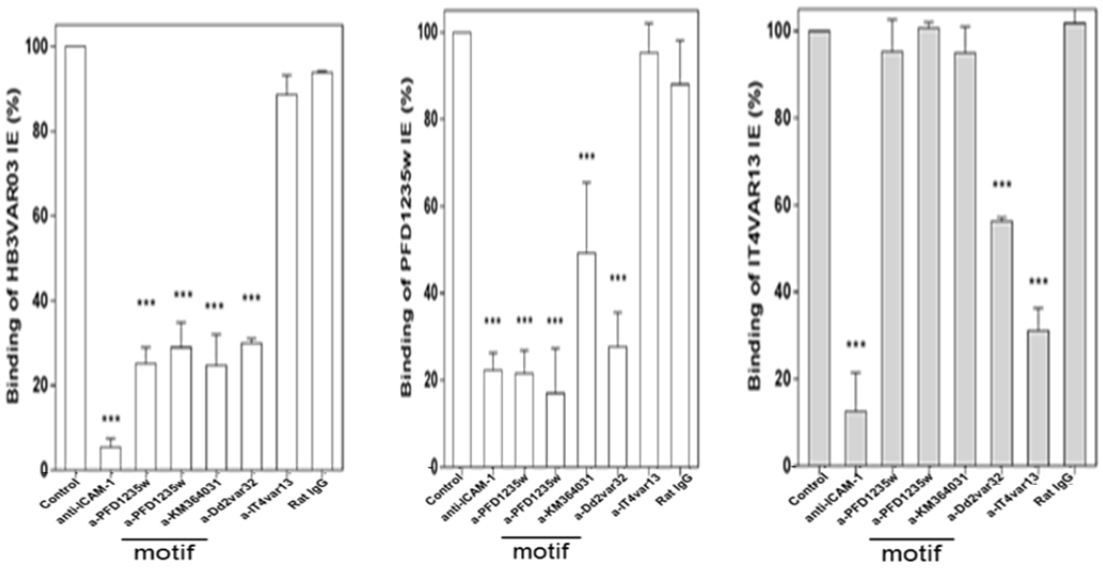


Figure 4.3: Inhibition of ICAM-1 binding to *P. falciparum* lab isolates by antibodies raised against motif DBLβ domains. Adhesion inhibition of ICAM-1 binding of HB3VAR03 IE (left), 3D7 PFD1235w IE (centre), and IT4VAR13 IE (right) to ICAM-1 under physiological flow (1 dyn/cm^2) in the presence of anti-ICAM-1 antibodies, rat affinity purified anti-PFD1235w_D4 IgG, anti-PFD1235w_motif IgG, anti-KM364031_motif IgG, anti-Dd2VAR32_motif IgG, anti-IT4VAR13 and control IgG. At least three independent experiments performed in triplicates are reported. Data are represented as mean± S.D. *** indicate significance, p <0.01.

4.4.5 ICAM-1 adhesion-inhibition of recombinant motif domains and *P. falciparum* isolates by motif-specific peptide antibodies

Supplemental experiments aimed to test the binding-inhibitory functionality of antibodies against the motif peptide (minimum ICAM-1 binding region) were performed. Briefly, rats were immunised with either the PFD1235w motif peptide alone or combined with a Dd2VAR32 motif peptide. The resulting antisera from either the single peptide or the double peptide immunisations were tested for their ability to inhibit ICAM-1 binding in ELISA as described before. The data showed that the motif peptide antisera from each immunised rat interfered with the ICAM-1 binding of a broad selection of motif-containing DBLβ domains to recombinant ICAM-1. This inhibition was increased when antisera from rats immunised with both

peptides was used. Conversely, the peptide antibodies did not inhibit ICAM-1 binding of non-motif containing IT4VAR13 and IT4VAR16 DBLβ domains (Figure 4.4).

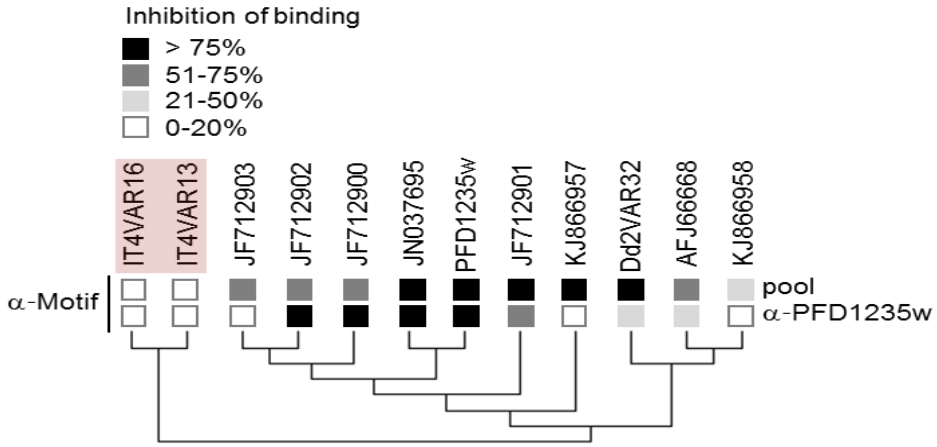


Figure 4.4: Inhibition of ICAM-1 binding by motif peptide-specific antibodies to motif DBLβ proteins. Raised antisera from rats immunised with one (PFD1235w) or two (pool: 3D7 PFD1235w and Dd2VAR32) motif peptides tested in ELISA inhibited ICAM-1 binding of several motif-containing DBLβ but were not effective at inhibiting non-motif DBLβ binding (IT4VAR16, IT4VAR13) to ICAM-1.

The capacity of peptide antisera to disrupt IEs capable of adhering to ICAM-1 and EPCR concomitantly was assessed. IEs selected to express PFD1235w or HB3VAR03 were incubated with either single peptide or double peptide antisera (pool) and subsequently allowed to bind recombinant ICAM-1 receptor coated on a microslide under flow conditions. Similar experiments were done with IT4VAR13 IE. Antibodies raised against single peptide markedly interfered with the dual binding parasite isolates of both 3D7 PFD1235w IE (homologous) and HB3VAR03 IEs (heterologous) to ICAM-1 and more evidently, a greater binding inhibition was recorded when the motif peptide pool antisera was used. Contrastingly, both the single or pool peptide

antisera were not capable of disrupting the ICAM-1 binding of IT4VAR13 parasite isolate, a CD36 and ICAM-1 dual binder (Figure 4.5A).

In addition, peptide antisera were incubated with HB3VAR03, 3D7 PFD1235w or IT4VAR13 selected IEs to determine whether they reacted with the PfEMP1 expressed on the surface of these IEs. Data from immunofluorescence assays showed that the peptide antisera only recognised the surface of motif-expressing HB3VAR03 and 3D7 PFD1235w IE and not the non-motif IT4VAR13 control (Figure 4.5B).

Similar flow adhesion-inhibitory experiments were carried out using *P. falciparum* isolates obtained from Ghanaian (n=14) and Tanzanian patients (n=19). A total of 22 parasites isolates (3UM, 14 SM and 5CM) showed adhesion to ICAM-1. Motif-specific peptide antisera inhibited a considerable number (11/22) of ICAM-1 binding parasite isolates, notably, one from a child with UM, six with SM, and four with CM at variable degrees. Adhesion blocking activity was around 25-40 % for three of the parasite isolates, while 45-68 % was recorded for the other eight isolates. Taken together, the data showed that the children were infected with IEs that harboured the motif (Figure 4.5C) and more importantly, vaccination with motif peptides induced cross-reactive antibodies that inhibited the binding of four out of five *P. falciparum* isolates obtained from paediatric CM patients.

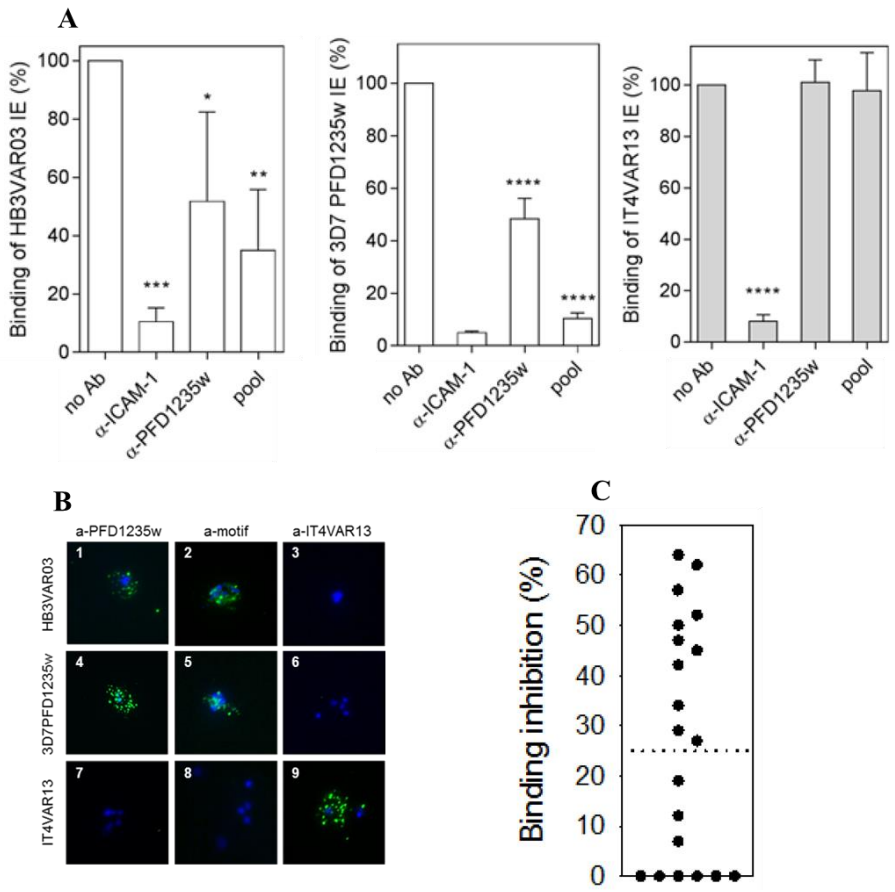


Figure 4.5: Adhesion inhibition of ICAM-1 binding of *P. falciparum* parasites by motif peptide-specific antibodies. (A). Adhesion inhibition of ICAM-1 binding of HB3VAR03 IE (top), 3D7 PFD1235w IE (centre), and IT4VAR13 IE (bottom) under flow conditions (1dyn/cm^2) in the presence of anti-ICAM-1 antibodies, rat plasma anti-PFD1235w_motif and rat plasma anti-PFD1235w/ Dd2VAR32_motif (pool). At least three independent experiments performed in triplicates are reported. Data are represented as mean ± S.D. * indicates significance **(B)**. Antibodies raised against the motif peptides were incubated with laboratory isolates namely HB3VAR03 IE (1-3), 3D7 PFD1235w IE (4-6) and IT4VAR13 IE (7-9). Peptide antibodies recognised the surface expression of PfEMP1 only in HB3VAR03 IE (1-3), 3D7 PFD1235w IE (4-6) using immunofluorescence assay (representative image). Antibodies used were anti-PFD1235w DBLβ_D4, anti-IT4VAR13 DBLβ_D4 and anti- PFD1235w/ Dd2VAR32_motif (pool). **(C)**. Adhesion inhibition of ICAM-1 binding of *P. falciparum* field isolates (n=20) by motif specific antibody pool. Inhibition assay was done only if parasite isolate bound to ICAM-1 was ≥ 10 IE/mm^2 in the adhesion assay. Dotted line represents 25 % inhibition cut-off. A phylogenetic tree indicating the relatedness of the different domains is shown to the extreme left on figure A. Blue shading indicates non-motif containing DBLβ. Level of inhibition is shown as: black (>75%), dark gray (50-75%), gray (20-50%), and white (<20%).

4.5 Discussion

The protective role of naturally-acquired antibodies against *P. falciparum* infection has long been recognised (Sabchareon *et al*., 1991; Cohen *et al*., 1961). Earlier studies have demonstrated that the surface exposed PfEMP1 antigen is the principal target of protective immunity since antibodies directed against this antigen have been linked with a decreased risk of developing clinical malaria (Chan *et al*., 2012; Nielsen *et al*., 2002; Ofori *et al*., 2002). Additionally, available evidence indicates that increased exposure to *P. falciparum* leads to the production of a broadly effective PfEMP1 antibody response, resulting in cross-reactive protection (Cham *et al*., 2009; Mackintosh *et al*., 2008; Krause *et al*., 2007). Thus, on one hand, the data from these studies provide a strong rationale for the development of an anti-PfEMP1 based vaccine. On the other hand, the enormous diversity of PfEMP1 found within and between different parasite populations makes them unattractive candidates. Despite this widespread diversity, there is increasing evidence suggesting that there may be structurally conserved epitopes that may be considered potential vaccine targets or constituents (reviewed in Bull and Abdi, 2016).

Consistent with these observations, in chapter three, it was demonstrated that CM is precipitated by group A PfEMP1 expressing IEs with dual and simultaneous binding affinity for EPCR and ICAM-1. These IEs were found to contain a structurally conserved ICAM-1 binding DBLβ motif capable of eliciting cross-reactive antibodies. Given its association with CM development, this study investigated in detail the antibody responses against an array of group A PfEMP1 with the motif-containing ICAM-1 or non-motif DBLβ recombinant proteins. The study further explored the potential utility of motif containing DBLβ domains and peptides to mount cross-reactive antibody responses interfering with the ICAM-1 binding of several

recombinant DBLβ domain constructs as well as IEs expressing motif-containing PfEMP1.

Antibody reactivity to group A PfEMP1 recombinant DBLβ proteins with an ICAM-1 binding motif or non-motif was measured in 79 plasma samples obtained from children with SM or UM. Overall, there was a striking degree of variability in antibody responses to the DBLβ recombinant proteins in the different plasma donors, with higher titres and broader antibody recognition observed in some donors (Figure 4.1).

Most studies have previously demonstrated that host age is a major determinant during the acquisition of naturally protective partial immunity in endemic populations, possibly resulting from frequent exposures to *falciparum* parasites (Joergensen *et al*., 2007; Magistrado *et al*., 2007; Ofori *et al*., 2002; Bull *et al*., 2000). This is further supported by the fact that the heaviest toll of malaria infections are carried by children younger than 5 years, particularly in regions of ongoing transmission (Gupta and Day, 1994; Greenwood *et al*., 1991). In keeping with this observation, children were grouped according to age to compare antibody responses between the groups. An overall trend of an age-related build-up of antibody repertoire to both motif and non-motif DBLβ proteins was evident and this trend resonated with what had been previously reported for other PfEMP1-based studies (Cham *et al*., 2010; Cham *et al*., 2009; Magistrado *et al*., 2007; Ofori *et al*., 2002). The mean anti-DBLβ response increased significantly from the four year old or younger age group to the more than four years old group for both types of DBLβ domains. Also, most plasma samples from children recorded markedly higher responses to non-motif DBLβ proteins compared with responses towards motif proteins only in the younger age group (Figure 4.1). A possible explanation for this observation may be that the children had

limited exposure to IEs expressing motif-containing DBLβ domains. There was however, no difference in antibody responses to both motif and non-motif proteins in the older age groups, suggesting that natural immunity to motif proteins is acquired later in comparison with non-motif proteins.

When patients were stratified according to disease severity, no appreciable difference in antibody reactivity was detected between SM and UM in both DBLβ protein groups within the same age group. However, some earlier studies have observed higher anti-PfEMP1 responses in UM patients and thus suggested that may have prevented the development of severe forms of malaria (Duffy *et al*., 2016; Rovira-Vallbona *et al*., 2012; Mayor *et al*., 2009; Tebo *et al*., 2002). The data presented in chapter three clearly showed that IEs that transcribed group A motif-containing ICAM-1 binding PfEMP1 were exclusively involved in CM development. This study however, was not able to obtain plasma from CM patients and that may possibly account for the observed results. In keeping with the finding of chapter three, it would have thus been predicted that significantly reduced motif antibody levels would be quantified in CM individuals in comparison with non-cerebral SM or UM.

Earlier studies have demonstrated that immune plasma from individuals in malaria-endemic areas contained IgG inhibitory of ICAM-1 binding (Lennartz *et al*., 2017; Bengtsson *et al*., 2013b). Consistent with that, a plasma pool generated from the study donors and affinity purified on motif domain was able to inhibit ICAM-1 binding of a heterologous motif DBLβ domain. The results presented here likely fits into the proposed mechanism where anti-PfEMP1 antibodies offer clinical protection by blocking tissue-specific sequestration, resulting in non-adherent parasites effectively destroyed by the spleen and resulting in a considerable reduction in parasite load (Dodoo *et al*., 2001). Thus, it may be possible to develop a vaccine based on this

ICAM-1 binding motif to prevent CM by circumventing parasite sequestration in the brain.

A vaccine based on the motif involved in ICAM-1 binding must be able to evoke a strong anti-adhesive immune response. Consistent with this notion, the potential of using individual DBLβ domains or motif peptides representing the group A ICAM-1 binding site to trigger cross-reactive and anti-adhesive IgG was investigated. Rat immunisations were done with either motif peptides (ICAM-1 binding epitope only) or motif-containing DBLβ domains. Significantly, rat antisera raised against motif peptides effectively blocked a broad selection of DBLβ domains involved in ICAM-1 binding (Figure 4.4).

It is essential that a vaccine based on the motif is capable of inhibiting IE adhesion regardless of the origin of the parasite strain. Notably, antisera generated from only two motif-specific peptides were effective at blocking significantly adhesion of ICAM-1 binding of several malaria parasites including two laboratory *falciparum* isolates expressing motif-specific PfEMP1 (Figure 4.5A) and *falciparum* parasites isolated from four out of five paediatric CM patients (Figure 4.5C).

Some limitations were identified in this study. The number of plasma samples used for screening the antibody response was inadequate and thus did not provide enough statistical power to detect profound differences between SM and UM. The study could also not evaluate motif reactivity in plasma from CM pateints because none of the plasma donors had CM complications. However, these limitations to some extent were counteracted by the inclusion of additional experiments with the parasite isolates from CM patients which validated the cross-inhibitory functionality of antibodies targeting DBLβ motif and thus demonstrated the importance of the motif in CM. Further

investigations will have to be carried out on a large number of samples particularly from CM patients, though it is recognised that it has become increasingly difficult to access samples from CM patients.

It is widely accepted that the clinical epidemiology of malaria including transmission seasons, endemicity and intensity play decisive roles in shaping the development of immunity (Cham *et al*., 2009; Mayor *et al*., 2009; Giha *et al*., 2000; Rogier *et al*., 1999; Snow *et al*., 1994). Thus, future studies would have to evaluate the antibody responses against this motif using samples from different geographic regions while evaluating these influential factors to determine if replicative responses can be generated and that would be in line with its consideration as a potential global vaccine candidate. In addition, future longitudinal studies would be crucial for establishing the involvement of these antibodies in the development of immunity. Notwithstanding, this study showed that antibodies that are cross-adhesion inhibitory of a large set of motif DBLβ domains and IEs expressing native PfEMP1 associated with CM can be experimentally induced, in addition to being acquired following natural *falciparum* infections. A motif-specific peptide (representing the ICAM-1 binding epitope) known to be involved with paediatric CM elicits cross-reactive IgG that can functionally protect against CM, making it a promising vaccine candidate that should further be investigated.

CHAPTER FIVE

MARKERS OF INFLAMMATION AND ENDOTHELIAL ACTIVATION IN GHANAIAN CHILDREN WITH MALARIA

5.1 Introduction

The aetiology of SM remains difficult to explain, notably why a small fraction of infected children succumb to severely fatal complications. The host immune system is believed to be one of the major contributors to severe disease development (reviewed in Clark *et al*., 2006; van der Heyde *et al*., 2006).

During the erythrocytic cycle, the presence of the malaria parasites, accompanied by the release of parasite toxins into the blood circulation, directly stimulates immune cells to secrete both pro- and anti-inflammatory cytokines which are important for combating the infection and hence offering protection from malaria (reviewed in Schofield and Grau, 2005; Clark and Schofield, 2000). However, overproduction of pro-inflammatory cytokines could have detrimental consequences for the host. Indeed, circulating levels of TNF-α, IL-1 and high mobility group box 1 (HMGB1) protein present in the serum of malaria patients have been reported to correlate significantly with malaria severity (Alleva *et al*., 2005; Kwiatkowski *et al*., 1990; Grau *et al*., 1989). An imbalanced inflammatory response is thought to contribute to disease severity by increasing the expression level of endothelial surface adhesion molecules such as ICAM-1 (Turner *et al*., 1994; Ockenhouse *et al*., 1992), thereby increasing IE sequestration to endothelial cells in microvascular beds resulting in flood flow impediment (reviewed in Miller *et al*., 2002).

The increased sequestration of IEs together with a dysregulated host inflammatory response, are believed to activate the endothelium and contributes to the increased expression of adhesion molecules and microvascular leakage, exacerbating the

pathological processes of malaria (reviewed in Liles and Kain, 2014; Cunnington *et al*., 2013b; Kim *et al*., 2011). Indeed, immunohistochemical evaluations provide evidence of endothelial cell activation during malaria (Silamut *et al*., 1999; Turner *et al*., 1998; Turner *et al*., 1994). During endothelial activation, some of these endothelial receptors including EPCR and ICAM-1 are shed as soluble forms into circulation possibly through the action of metalloproteinases (Qu *et al*., 2007; Fiore *et al*., 2002; Xu *et al*., 2000) and these may serve as biomarkers of inflammation and endothelial activation. Indeed, studies have reported increased levels of these soluble molecules in acute malaria suggesting that they form a critical component or markers of the onset of disease pathology (Moussiliou *et al*., 2015; Adukpo *et al*., 2013; Cserti-Gazdewich *et al*., 2010). Furthermore, the activated endothelial cells can fuse with Weibel Palade bodies and facilitate the rapid release of their contents, including, von-Willebrand factor and Ang-2 directly into circulation (Fiedler and Augustin, 2006). High plasma concentrations of Ang-2 were found to correlate with death in individuals infected with *falciparum* malaria (Prapansilp *et al*., 2013; Yeo *et al*., 2008).

The present study quantified plasma levels of HMGB1, Ang-2, sEPCR and sICAM-1 in paediatric Ghanaian with malaria on the day of clinical presentation (admission samples, week 0) and two weeks following treatment (convalescent samples, week 2). This was to examine whether there are alterations in the levels of these soluble markers during the acute phase and after resolution of the infection. This panel of biomarkers were investigated based on their role in disease pathogenesis and no study has assessed their kinetics concurrently in Ghanaian children with malaria.

5.2 Hypothesis and specific objectives

High levels of endothelial markers of activation are associated with disease severity

Specific objective

1. To evaluate the levels of biomarkers of activation and inflammation in the plasma of acute and convalescent children with UM and SM.

5.3 Methods

5.3.1 Study outline

This study was carried out within the frame of a broader study which aimed to build malaria vaccine capacity in Ghana. All ethics-related protocols and permit to conduct the study were granted by the institutional review board of Noguchi Memorial Institute for Medical Research and the Ethical Review Committee of the Ghana Health Service (file GHS-ERC 08/05/14). Participation in the study was voluntary and the patients were enrolled after parents or guardians signed the written informed consent. Patients were treated by adhering to the standard guidelines of the Ministry of Health in Ghana. Children (<12 years of age) reporting with malaria, or non-malarial febrile illness to the Hohoe Municipal Hospital, Ghana were enrolled into the study during the 2015 and 2016 malaria transmission seasons. Malaria transmission in this township occurs throughout the year with marked seasonal peaks after the major rains in June. On average, inhabitants are exposed to approximately 65 infectious bites per person per year (Kweku *et al*., 2015; Kweku *et al*., 2008). A detailed description of the study site can be found in chapter four section 4.3.1.

The enrolled children immediately underwent clinical investigations and blood samples were taken for disease diagnosis (parasite evaluation and haematology) and

research purposes. Malaria was diagnosed by a rapid diagnostic kit and by light microscopy from Giemsa-stained thick and thin peripheral blood smears. The children were then placed into categories following the WHO criteria for malaria diagnosis (WHO, 2000) as described before (see section 4.3.2). Febrile children (FC) ($\geq$ 37.5 °C) reporting to the hospital who tested negative via a rapid diagnostic test and microscopic investigation were enrolled. Age-matched healthy community school children (CC) who tested negative for malaria via a rapid diagnostic test and microscopic investigation with no previous malaria treatment in the past two weeks before enrollment were also included in the study.

5.3.2 Sample collection and processing

Blood was collected into EDTA (6 mL) and Heparin (4 mL) vacutainers (Beckon Dickson, Germany) from consenting children on the day of admission (week 0) and on week two following initial presentation (convalescent patients). For healthy community controls, blood samples were collected at only one time point. Blood was immediately centrifuged to separate plasma from pelleted erythrocytes. Plasma samples were aliquoted into cryovials and stored immediately in a -80 °C freezer. The samples were then shipped on dry ice to Copenhagen and further kept in a freezer at -80 °C until they were used for quantifying inflammation markers by ELISA.

5.3.3 ELISA for markers of Inflammation/endothelial activation

All reagents and samples were allowed to equilibrate to room temperature prior to analysis. Plasma samples, controls and standards were assayed in duplicates. Measurements for the markers of inflammation or endothelial activation were carried out on plasma samples collected at admission (week 0), week two following initial

presentation (week 2) and recovery, as well as community controls. All biomarkers were quantified using commercial kits employing a sandwich ELISA and carried out at room temperature. Concentrations were determined by interpolation of a four-parameter-fit standard curve generated for the various markers.

5.3.3.1 Quantitative determination of sICAM-1 in human plasma samples

The plasma concentration of soluble ICAM-1 (sICAM-1) was quantified using a commercial ELISA kit (DCIM00; R&D Systems, USA) according to the manufacturer's instructions. Precoated microplate strips with human ICAM-1 non-allele-specific monoclonal antibody were incubated with standards (0.625 ng/mL-40 ng/mL) diluted in calibrator diluent RD5P (1:5); 100 µL/well) and samples (1:20 in calibrator diluent RD5P (1:5); 100 µL/well) for 1 h on a horizontal orbital microplate shaker at 500 rpm ± 50 rpm. The wells were aspirated, washed four times (400 µL wash buffer/per well) and padded dry on clean paper towels. The plates were incubated with human ICAM-1 non-allele-specific conjugate (200 µL/well) for 1 h on a shaker which was followed by washing (400 µL/well; four times). An aliquot of 200 µL of substrate solution was added to each well and incubated for 30 min in the dark. Stop solution (2N H_2SO_4; 50 µL) was added to all wells to halt the colour reaction and the plates were read at 450 nm and 540 nm using a VERSAmax reader (Molecular Devices, USA).

5.3.3.2 Quantitative determination of sEPCR in human plasma samples

The assay was carried out using a commercial kit (dy2245; R&D Systems, USA) and following the manufacturer's protocols. Plates were coated with the capture antibody (100 µL; 800 ng/mL in PBS) and incubated overnight at room temperature. Plates

were washed three times with 400 µL per well with wash buffer (0.05 % Tween in PBS) and blocked with reagent diluent (300 µL; 1 % BSA in PBS) for 1 h. Diluted plasma samples (1:20, in reagent diluent; 100 µL) and standards (0.313 ng/mL-20 ng/mL in reagent diluent; 100 µL) were added to wells and incubated for 2 h. The plates were aspirated, washed three times and incubated with detection antibody (1:200; 100 µL) for 2 h. Following washing, plates were incubated with streptavidin conjugated to horseradish peroxidase (1:200; 100 µL) for 20 min in the dark. Washed plates were subsequently incubated with substrate solution (100 µL) in the dark for another 20 min and reaction curtailed by the addition of 50 µL of stop solution (2N H_2SO_4) to each well. Plates were read using a VERSAmax plate reader (Molecular Devices, USA) at 450 nm and 540 nm to account for background correction.

5.3.3.3 Measurement of Ang-2 concentration in human plasma samples

The plasma concentration of Ang-2 was quantified using a commercial ELISA kit (DANG20 duo set; R&D Systems, USA) according to the manufacturer's instructions. Precoated microplate strips with a monoclonal antibody specific for human Ang-2 were allowed to equilibrate to room temperature and the assay diluent, RD1-76 reagent (100 µL/well), was added to the plates. Plates were incubated with standards (46.9 pg/mL-3000 pg/mL in reagent diluent; 50 µL) and samples (1:5 in reagent diluent; 50 µL) for 2 h on a horizontal orbital microplate shaker at 500 rpm ± 50 rpm. The wells were aspirated, washed four times with 400 µL of wash buffer per well and blotted on clean paper towels. Human Ang-2 conjugate (200 µL/well) was added to each well and incubated for 2 h on a plate shaker. Washed plates were subsequently incubated with 200 µL of substrate solution per well for 30 min in the dark. The reaction was stopped with 50 µL/well of stop solution (2 N H_2SO_4) and the OD was

determined at 450 nm and 540 nm using a VERSAmax reader (Molecular Devices, USA).

5.3.3.4 Measurement of HMGB1 concentration in human plasma samples

Plasma HMGB1 was measured according to the manufacturer's protocol (ST51011; IBL International, Germany). Reagent diluent (100 µL) was added to each well of precoated microtitre plates. Plates were incubated with 10 µL of standards (2.5 ng/mL-80 ng/mL in reagent diluent) and plasma samples (10 µL) for 22-24 h at 37 ^{0}C. Plates were washed five times with 300 µL per well with wash buffer (< 0.5% Tween in PBS). In each well, 100 µL of enzyme conjugate was added and plates incubated for 2 h at 25 ^{0}C. Washed plates were further incubated with 100 µL per well with colour reagents (ready to use TMB and 0.005 M H_2O_2) for 30 min in the dark. The reaction was stopped by the addition of 50 µL/well of stop solution (0.35 M H_2SO_4) and the plates read at 450 nm using a VERSAmax plate reader (Molecular Devices, USA).

5.3.4 Data analysis

Statistical analyses were performed using the SigmaPlot13 (Systat Software Inc, United Kingdom). Clinical information and soluble marker data on study groups were summarised as median and inter quartile range (25 %; 75 % quartiles). Continuous variables were analysed using the Kruskal-Wallis Rank Sum Test with Dunn's multiple comparison test. Comparison between admission and convalescent samples were analysed using the Mann-Whitney U Test. Statistical significance was set at $p \leq 0.05$.

5.4 Results

5.4.1 Baseline information on study groups

The demography and clinical information of enrolled children are summarised in Table 5.1. A total of 191 children were enrolled in this study. They included 41 children with SM (4 CM, 3 SA, 1 CM/SA, 33 hyperparasitaemia), 69 children with UM, 13 children with FC and 68 CC. In the SM group, the majority were males whereas, in the UM group, gender was uniformly spread. The median ages were comparable among the study participants. Also, median parasitaemia was not statistically different between malaria groups. Expectedly, higher median Hb levels were detected in healthy CC than in SM ($p < 0.001$) and UM ($p = 0.045$), with higher levels in UM compared to SM ($p < 0.001$; Kruskal-Wallis, Dunn's Test). There was also no significant difference between either FC and CC or FC and UM (Kruskal-Wallis, Dunn's Test). Median temperature recordings were markedly lower in CC than in SM ($p < 0.001$), UM ($p < 0.001$) and FC ($p < 0.001$; Kruskal-Wallis, Dunn's Test).

Table 5.1: Baseline clinical profile and the levels of soluble markers of inflammation and endothelial activation.

Variable	SM(n=41)	UM(n=69)	FC(n=13)	CC(n=68)
Age(years)	4.45(2.45-5.58)	3.85(2.70-6.75)	2.90(2.90-2.90)	5.00(3.00-6.00)
Male (%)	28(68.3 %)	34(49.3 %)	11(84.6 %)	29(42.6 %)
Haemoglobin(g/dL)	7.80(7.10-9.10)	10.00(9.25-11.25)	11.20(9.65-11.95)	11.40(9.80-12.20)$^{\alpha}$
Temperature (^{o}C)	38.50(37.50-39.60)	39.00(37.80-39.50)	38.60(38.35-39.30)	36.35(36.00-36.70)$^{\beta}$
Parasites*1000/μL	52.51(17.56-130.67)	38.66(18.80-78.18)	NA	NA
sICAM-1 (ng/mL)	876.36(770.47-982.28)	790.97(685.78-958.86)	623.76(499.70-818.35)	339.24(288.97-399.03)
sEPCR(ng/mL)	16.27(11.52-22.51)	16.81(11.48-23.76)	11.74(8.36-22.99)	4.39(2.60-7.56)
Ang-2 (pg/mL)	5926.30(2941.35-7895.76)	3873.22(2786.54-6008.00)	2379.39(1727.87-3641.16)	1474.67(1123.91-2095.18)
HMGB1(ng/mL)	9.66(6.48-19.30)	10.80(5.65-18.87)	4.84(3.03-12.63)	4.96(3.03-7.94)

Data are presented as median (interquartile range) except male gender (%). Groups were compared using Kruskal-Wallis Rank Sum Test followed by Dunn's Post Hoc Test for multiple comparisons. Mann-Whitney U Test was used to compare parasite density between UM and CM. α-indicates that the median Hb levels were significantly higher in CC than in SM ($p < 0.001$) and UM ($p = 0.045$). β-indicates that the median temperature was significantly lower in CC than in SM, UM and FC ($p < 0.001$). UM = uncomplicated malaria, SM = severe malaria (includes cerebral malaria (CM; n = 4), severe malarial anaemia (SA; n = 3), CM/SA (n = 1), hyperparasitaemia (n = 33)), FC = febrile control (n=13) and CC = community control (n=68), NA=not applicable.

5.4.2 Soluble ICAM-1 (sICAM-1) levels

Data on sICAM-1 were evaluated in admission samples (week 0) and in follow-up convalescent samples (week two) of SM, UM, FC and CC study participants and the data are summarised in figure 5.1. In week 0, significantly elevated levels were recorded for malaria groups (SM=876.36 ng/mL; UM=790.97 ng/mL) or FC group (623.76 ng/mL) in comparison with CC group (339.24 ng/mL; $p < 0.001$, Kruskal-Wallis, Dunn´s Test). Although the median sICAM-1 concentration was higher in the SM group compared with UM, the difference was not statistically significanct (Figure 5.1A). Levels of sICAM-1 decreased in convalescent samples (week two) when compared with week 0 levels in the malaria groups (UM week 0 versus UM week two; $p < 0.001$; SM week 0 versus SM week two; $p < 0.001$; Mann-Whitney U Test) [Figures 5.1B and 5.1C]. There was no difference in sICAM-1 levels among the different age categories in both SM and UM ($p > 0.05$) [Appendix D; Dunn's Test].

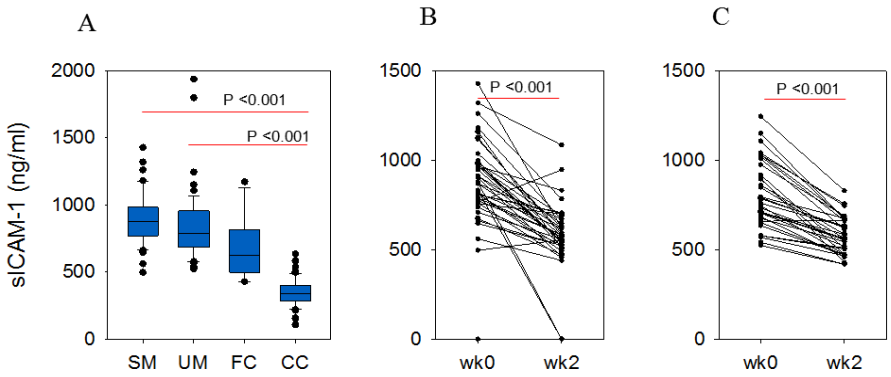


Figure 5.1: Median sICAM-1 levels among study groups. (A) sICAM-1 levels were assessed by ELISA in children with SM (41), UM (69), non-malarial febrile illness (FC; 13) and healthy community controls (CC; 68) on admission day (week 0). Data are presented as box plots with median and with 25th and 75th percentile distribution of data. Whiskers represent minimum and maximum values with outliers. **(B-C)** sICAM-1 levels on week 0 compared with week two samples in both SM **(B)** and UM **(C)**. Each horizontal line represents the data points for each individual measurement. Multiple variables were analysed using the Kruskal-Wallis Rank Sum Test with Dunn's Multiple Comparison Test. Comparisons between two variables were done using Mann-Whitney U Test. Wk 0 = week 0; wk2 = week two

5.4.3 Soluble EPCR (sEPCR) levels

Quantification of the sEPCR levels in the study groups in admission samples (week 0) showed that malaria patients and children in the FC group generally had higher levels of sEPCR (SM = 16.27 ng/mL; UM = 16.81; ng/mL; FC = 11.74 ng/mL, $p < 0.001$) compared with the healthy CC group (4.39 ng/mL; Kruskal-Wallis, Dunn´s Test). There was no significant difference between UM and SM patients (Figure 5.2A). Following two weeks after initial presentation, sEPCR levels decreased significantly in comparison with week 0 levels in the SM group ($p < 0.001$; Mann-Whitney U Test; Figure 5.2B). A similar trend was recorded for the UM group when week 0 was compared to week two ($p < 0.001$, Mann-Whitney U Test) [Figure 5.2C]. There was no significant difference in sEPCR levels among the different age groups in both SM and UM ($p > 0.05$; Dunn's Test) [Appendix D]

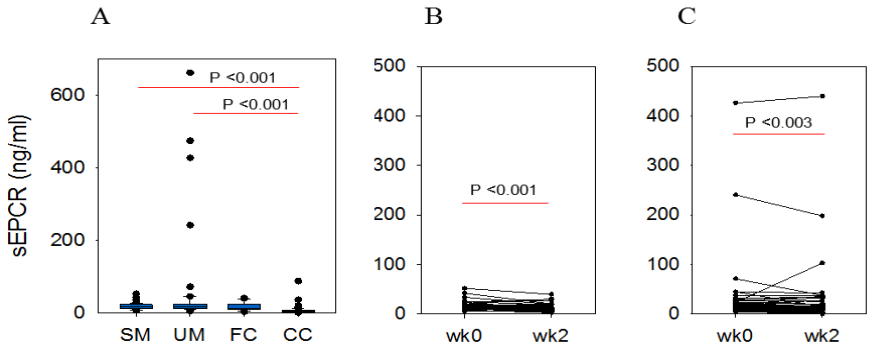


Figure 5.2: Median sEPCR levels among study groups. (A) sEPCR levels were assessed by ELISA in children with SM (41), UM (69), non-malarial febrile illness (FC; 13) and healthy community controls (CC; 68) on admission day (week 0). Data are presented as box plots with median and with 25th and 75th percentile distribution of data. Whiskers represent minimum and maximum values with outliers. **(B-C)** sEPCR levels on week 0 compared with week two samples in both SM **(B)** and UM **(C)**. Each horizontal line represents the data points for each individual measurement. Multiple variables were analysed using the Kruskal-Wallis Rank Sum Test with Dunn's Multiple Comparison Test. Comparisons between two variables were done using Mann-Whitney U Test. Wk 0 = week 0; wk2 = week two

5.4.4 Plasma Ang-2 levels

Plasma Ang-2 levels were evaluated in week 0 and in week two plasma samples of children in the SM, UM, FC and CC study groups and data are summarised in figure 5.3. In week 0 samples, children with malaria (SM, UM) recorded significantly higher Ang-2 median levels (SM = 5926.23 pg/mL; UM = 3873.22 pg/mL) when compared with the CC group (1471.67 pg/mL, p $\leq$ 0.001; Dunn's Test; Figure 5.3A). The median Ang-2 levels were markedly lower in the FC group (2379.39 pg/mL) when compared with the SM group (p = 0.039; Dunn´s Test). Median Ang-2 concentration did not differ significantly between SM and UM (p > 0.05, Dunn's Test; Figure 5.3A). When Ang-2 levels in the malaria group were compared between week 0 samples and week two samples, the median levels were significantly reduced in the latter (p < 0.001, Mann-Whitney U Test; Figure 5.3B-5.3C). Comparable median levels of plasma Ang-2 were observed among the different age categories in both malaria groups (p > 0.05; Kruskal-Wallis, Dunn´s Test; Appendix D).

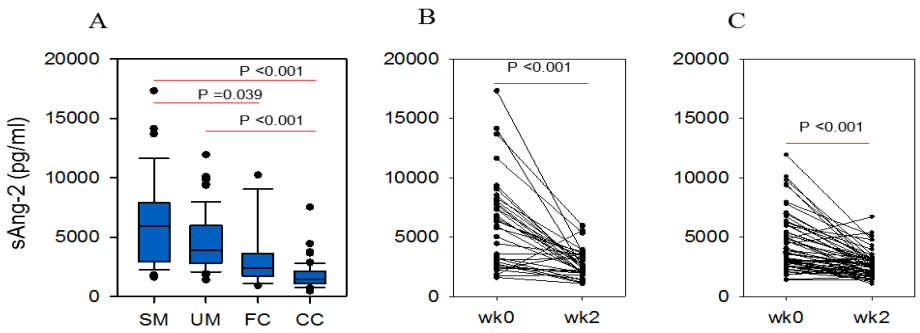


Figure 5.3: Median plasma Ang-2 levels among study groups. (A) Plasma Ang-2 levels were assessed by ELISA in children with SM (41), UM (69), non-malarial febrile illness (FC; 13) and healthy community controls (CC; 68) on admission day (week 0). Data are presented as box plots with median and with 25th and 75th percentile distribution of data. Whiskers represent minimum and maximum values with outliers. **(B-C)** Ang-2 levels on week 0 compared with week two convalescent samples in both SM **(B)** and UM **(C)**. Each horizontal line represents the data points for each individual measurement. Multiple variables were analysed using the Kruskal-Wallis Rank Sum Test with Dunn's Multiple Comparison Test. Comparisons between two variables were done using Mann-Whitney U Test. Wk 0 = week 0; wk2 = week two

5.4.5 Plasma HMGB1 levels

Plasma concentrations of HMGB1 were determined in admission samples and in convalescent samples (week two) and the data are summarised in figure 5.4. Median levels of plasma HMGB1 were significantly increased in SM and UM when compared to the healthy CC group (SM = 9.66 ng/mL; UM = 10.80 ng/mL; CC = 4.96 ng/mL; $p < 0.001$; Kruskal-Wallis, Dunn´s Test; Figure 5.4A) on admission day (week 0; Figure 5.4A). The levels were however comparable between SM and UM in admission samples ($p > 0.05$ Dunn's test; Figure 5.4A). In week two convalescent samples, plasma HMGB1 levels remain high in both malaria groups similar to levels reported for week 0 ($p > 0.05$; Mann-Whitney U Test) [Figures 5.4B and 5.4C]. There was no difference in plasma HMGB1 levels among the different age groups in both malaria groups (Appendix D; Dunn's Test).

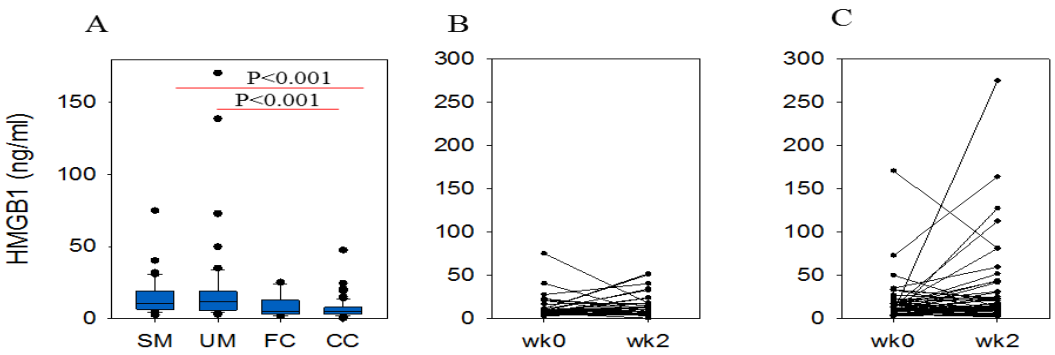


Figure 5.4: Median plasma HMGB1 levels among study groups. (A) Plasma HMGB1 levels were assessed by ELISA in children with SM (41), UM (69), non-malarial febrile illness (FC; 13) and healthy community controls (CC; 68) on admission day (week 0). Data are presented as box plots and with median with 25th and 75th percentile distribution of data. Whiskers represent minimum and maximum values with outliers. **(B-C)** HMGB1 levels on week 0 compared with week two convalescent samples in both SM **(B)** and UM **(C)**. Each horizontal line represents the data points for each individual measurement. Multiple variables were analysed using the Kruskal-Wallis Rank Sum Test with Dunn's Multiple Comparison Test. Comparisons between two variables were done using Mann-Whitney U Test. Wk 0 = week 0; wk2 = week two

5.5 Discussion

This study quantified a panel of markers including sICAM-1, sEPCR, Ang-2 and HMGB1 in plasma samples; comparing the levels between children with peripheral smear-positive *P. falciparum* infections, aged control children with febrile non-malaria infections and healthy community control children. The finding that levels of all inflammatory and endothelial activation mediators measured were markedly elevated in children presenting with acute malaria in comparison with healthy community controls are in agreement with the notion that acute malaria is partly a disease of systemic inflammation (reviewed in Storm and Craig, 2014; Miller *et al*., 2013; Clark *et al*., 2006).

The adhesion molecule ICAM-1 is a cytokine-inducible glycoprotein expressed at low levels on diverse cells including resting endothelial cells (Gearing and Newman, 1993). The physiological role of ICAM-1 is to mediate leucocyte adhesion and trafficking to inflammatory sites by binding to the leucocyte function-associated antigen and Mac 1, which are expressed on leucocytes (Diamond *et al*., 1990; Simmons *et al*., 1988). Expression of ICAM-1 is elevated following endothelial cell activation by various stimuli including cytokines (Gearing *et al*., 1992; Becker *et al*., 1991). ICAM-1 is a well-recognised major receptor for the virulent malaria parasite protein PfEMP1, sequestering IEs in vital organs including the brain (reviewed in Chakravorty and Craig, 2005). Corroborating evidence comes from histological studies that show extensive accumulation of IEs localised with raised levels of ICAM-1 in the brain tissues (Silamut *et al*., 1999; Turner *et al*., 1994). Membrane-bound ICAM-1 can be cleaved from the cell surface leading to the detection of increased concentration of its soluble forms in plasma during infection and that appears to

correlate with disease severity (Adukpo *et al*., 2013; Cserti-Gazdewich *et al*., 2010; Tchinda *et al*., 2007; Elhassan *et al*., 1994; Jakobsen *et al*., 1994; Hviid *et al*., 1993).

In this study, sICAM-1 levels were found to be significantly higher in Ghanaian children with *falciparum* malaria than in comparable healthy community control children on admission days (Figure 5.1A), supporting and extending previous findings (Park *et al*., 2012; Turner *et al*., 1998; Hviid *et al*., 1993). Soluble ICAM-1 levels did not differentiate children with SM from those with UM although a trend of higher median sICAM-1 levels was detected in SM (Figure 5.1A). Although this observation is consistent with previous findings (Park *et al*., 2012; McGuire *et al*., 1996; Deloron *et al*., 1994), there are other studies that have reported higher levels of sICAM-1 in individuals with SM in comparison with UM or controls (Cserti-Gazdewich *et al*., 2010; Tchinda *et al*., 2007; Turner *et al*., 1998). The site of endothelial inflammatory reactions may be an important determinant for disease presentation. In keeping with that, children with CM may experience inflammatory reactions in brain endothelial cells whereas children with UM with increased levels of sICAM-1 may have inflammatory reactions in endothelial beds in other parts of the body. It is also possible that children diagnosed with UM with high levels of sICAM-1 reported here could have advanced to severe disease if they had not sought treatment. The raised levels of sICAM-1 in acute malaria may indicate an increased activation of endothelial cells likely resulting in increased receptor expression and subsequent shedding from the vascular endothelium and activated lymphocytes (Turner *et al*., 1998; McGuire *et al*., 1996).

Like ICAM-1, EPCR serves as a receptor for specific PfEMP1 variants and has been implicated in SM complications (Lau *et al*., 2015; Turner *et al*., 2013). EPCR is a 64 kDa type 1 transmembrane glycoprotein expressed on a wide variety of cells, with

particularly low expression on brain microvascular cells (Laszik *et al*., 1997). It is involved in the regulation of inflammation, coagulation as well as in generalised cytoprotective processes on endothelial cells by converting protein C to activated protein C [APC] (Rao *et al*., 2014; Griffin *et al*., 2012; Stearns-Kurosawa *et al*., 1996; Fukudome and Esmon, 1994). There is considerable overlap in the PfEMP1 and APC binding interfaces on EPCR and PfEMP1 shows a greater binding affinity for EPCR (Lau *et al*., 2015). A soluble form of EPCR (sEPCR) is found circulating in plasma of healthy individuals and exhibits comparable binding affinity for APC (Fukudome *et al*., 1996; Regan *et al*., 1996). The plasma levels of sEPCR in a steady state are thought to be influenced by variations in the genetic sequence of EPCR (Uitte de *et al*., 2004; Xu *et al*., 2000). Many studies have determined the levels of sEPCR in an attempt to link these levels with severity of many infectious diseases (Moussiliou *et al*., 2015; Medina *et al*., 2004; Uitte de *et al*., 2004). In malaria, although sEPCR was shown to mediate binding to IEs and block their adhesion to human brain microvasculature endothelial cells (Petersen *et al*., 2015), the reports on sEPCR and disease associations have been conflicting. One study showed that elevated levels positively correlated with severe disease (Moussiliou *et al*., 2015) whereas other studies found reduced levels in SM (Shabani *et al*., 2016; Hansson *et al*., 2015).

The present study showed that sEPCR levels were increased in malaria patients in comparison with healthy controls (Figure 5.2A), consistent with observations in other diseases characterised by inflammation. Notably, in sepsis, a condition with similar symptomatology to malaria, the consensus is that raised levels are detected in individuals with sepsis in comparison with healthy individuals (Kendirli *et al*., 2009; Liaw *et al*., 2004; Kurosawa *et al*., 1998). The findings reported here are partially consistent with a previous study on Beninese children where raised sEPCR levels

were recorded in SM (CM) with lower levels in convalescent children (Moussiliou *et al*., 2015). However, no significant differences were detected between children with SM and UM in this study which is at variance with the Beninese study where sEPCR clearly discriminated the two disease syndromes (Moussiliou *et al*., 2015). In other studies, reduced levels of sEPCR were detected in SM, a marked departure from this study (Shabani *et al*., 2016; Hansson *et al*., 2015; Moxon *et al*., 2013). The observed differences could be due to the fact that the present study did not investigate the role of EPCR genetic variants. Indeed, earlier studies have demonstrated a connection between the prevalence of the rs867186-GG genotype and SM, although this is not always consistent (Shabani *et al*., 2016; Hansson *et al*., 2015; Naka *et al*., 2014; Schuldt *et al*., 2014). Thus, this study cannot possibly rule out the influence of genetic differences on the steady-state levels and whether those levels predisposed some individuals to be more or less susceptible to severe disease, although a study in Ghana showed that genetic variations did not affect susceptibility to SM (Schuldt *et al*., 2014).

The Angiopoietin family of proteins and their Tie-2 receptors are well-recognised regulators of the endothelial barrier integrity. On one hand, Ang-1-mediated Tie-2 signalling maintains the endothelium in a steady state by suppressing inflammation. Ang-2 on the other hand competitively opposes the protective effects of Ang-1 by priming endothelial cells to become responsive to inflammatory cytokines, which destabilises the microvascular integrity resulting in increased vascular permeability (reviewed in de Jong *et al*., 2016; Fiedler and Augustin, 2006). The importance of an activated and dysfunctional endothelium in the pathogenesis of SM cannot be overemphasized as previous studies have unequivocally documented significantly

decreased levels of circulating Ang-1 and increased Ang-2 levels in severe patients (Conroy *et al*., 2010; Conroy *et al*., 2009; Lovegrove *et al*., 2009).

This study documented elevated plasma levels of Ang-2 in admission samples of SM and UM in comparison with healthy community control children (Figure 5.3). The data generated are compatible with prior studies that demonstrate altered Ang-2 levels in severe disease in comparison with healthy controls (Jain *et al*., 2011; Yeo *et al*., 2008). Yeo *et al.,* (2008) showed that Ang-2 is a superior marker of SM-related deaths compared to lactate in Indonesian adults. Furthermore, Ang-2 levels alone have been proven to be a clinically robust marker of disease severity or mortality in children (Conroy *et al*., 2012). In the present study, plasma Ang-2 levels could not discriminate children with SM from UM, although increased Ang-2 levels were detected in the former. Thus, the data point to the potential function of Ang-2 dependent endothelial activation in the early onset of malaria.

The HMGB1 protein is an abundantly expressed nuclear non-histone protein believed to be an important late-onset cytokine mediator of systemic inflammation (Wang *et al*., 1999a). It is actively secreted from stimulated immune cells or passively secreted from necrotic cells (Scaffidi *et al*., 2002; Andersson *et al*., 2000; Wang *et al*., 1999b). Once liberated from cells, HMGB1 triggers inflammatory responses via interaction with its numerous binding receptors which result in downstream pathways to activate immune cells and endothelial cells to secrete more cytokines including TNF-α, which in turn can induce more HMGB1 production (Clark *et al*., 2006; Park *et al*., 2006; Fiuza *et al*., 2003; Andersson *et al*., 2000). Thus, secretion of HMGB1 into the circulation forms a positive feedback loop, a process that sustains a long-term inflammatory state (Andersson and Tracey, 2011; Clark *et al*., 2006; Alleva *et al*., 2005). Evidence of the importance of HMGB1 in promoting inflammation comes

from sepsis, a condition with a similar clinical resemblance to malaria. These studies reported elevated levels of plasma HMGB1 that correlated with the degree of organ dysfunction and poor disease outcome (Sunden-Cullberg *et al*., 2005; Wang *et al*., 2001). The suggestive role of HMGB1 in modulating the outcome of malaria infection has also been demonstrated. Indeed, studies have reported significantly increased levels of plasma HMGB1 in fatally ill African children relative to healthy controls or those who survived from severe disease (Angeletti *et al*., 2013; Higgins *et al*., 2013; Alleva *et al*., 2005). However, none of these studies have examined the kinetics of HMGB1. Thus, information pertaining to HMGB1 levels post recovery is not available.

In this study, markedly increased levels of plasma HMGB1 were detected in malaria patients and febrile children compared with age-matched healthy community control children in admission samples [week 0] (Figure 5.4A). This confirms findings from earlier malaria studies (Angeletti *et al*., 2013; Alleva *et al*., 2005) as well as reports from bacterial sepsis and other acute systemic inflammation-related conditions (Ito *et al*., 2011; Sunden-Cullberg *et al*., 2005). The levels of plasma HMGB1 were found to be comparable among the three clinical groups suggesting that HMGB1 may be a marker of general inflammation. In a previous study carried out on Ugandan children with *falciparum* malaria, higher HMGB1 levels were used to discriminate between SM and UM in admission samples and the levels were subsequently used to predict a fatal outcome (Higgins *et al*., 2013), which is at variance with this study. Notwithstanding, the data presented here demonstrate that plasma levels of HMGB1 may not always correlate with disease severity or fatal outcome which is in accordance with previous reports (Angeletti *et al*., 2013; Angus *et al*., 2007; Gibot *et al*., 2007; Sunden-Cullberg *et al*., 2005).

Soluble EPCR and sICAM-1 levels were raised in both malaria and febrile control groups, which is in line with previous studies that implicate endothelial activation in many infections (Moxon *et al*., 2014; Wang *et al*., 2013a; Saposnik *et al*., 2012). The levels of all the soluble markers assessed in this study except HMGB1 markedly declined following treatment in convalescent patients when two weeks post-treatment samples were compared with admission samples (Figures 5.1B-C, 5.2B-C, 5.3B-C). This suggests that the alterations in the levels of the various soluble markers screened at disease presentation were likely influenced by the infection rather than host genetic variations. The plasma levels of HMGB1 however, were found to persist in both malaria groups in convalescent samples at two weeks post treatment (Figure 5.4B-C), a finding reported for the first time in malaria studies. A similar observation has been documented in previous sepsis studies where they showed that levels remain increased even one week after treatment (Angus *et al*., 2007; Sunden-Cullberg *et al*., 2005). The relevance of this prolonged elevation is not entirely known but it may suggest a central role of HMGB1 in both febrile conditions and in disease progression by possibly protracting the symptoms of the disease. This study cannot rule out the potential influence of genetic variations on the observed data and future studies involving more time points may provide more details on when levels decline in malaria patients.

A notable observation in this study was that all the markers of inflammation and endothelial activation assessed could not discriminate between SM and UM as documented in some studies. A possible explanation for this difference may be related to the study population and sample size. In most previous studies the study cohort included well-categorised CM cases (Adukpo *et al*., 2013; Conroy *et al*., 2012; Cserti-Gazdewich *et al*., 2010). In the present study, however, a majority of the children in

the SM group consisted of children with hyperparasitaemia (n=33) [Table 5.1]. Only four CM cases were detected during the sampling season, supporting the observation that SM incidences are markedly declining in endemic regions (WHO, 2016). Thus, our sample size possibly affected our ability to detect any statistical difference between the malaria groups. It is thus predicted that significantly elevated levels of the various markers would have been detected with enough representation of more fatal cases, since a pattern of higher soluble marker levels was observed in severe cases than those with uncomplicated cases.

Some limitations apart from the effect of sample size were identified. Previously, it had been shown that markers of endothelial activation remain elevated in malaria patients compared with healthy controls up to a month after resolution of infection, possibly resulting from accumulated vascular injury encountered after multiple malaria infections (Moxon *et al*., 2014). In this study, the healthy control group was only sampled at one time point. Hence the levels of soluble markers in convalescent samples could not be compared to the healthy control group and as such, this study cannot totally rule out if the declined levels observed in convalescent samples are significant. Furthermore, the levels of soluble markers were not evaluated in asymptomatic non-febrile children. Contributions from parasite density alone to endothelial activation remains to be determined and such data may have important consequences given that most individuals in endemic populations are asymptomatic.

It is well accepted that SM pathology is complex, comprising both parasite cytoadhesion and host inflammatory processes. Although it is entirely unclear which of the factors initiates the pathological process, it is highly plausible that both processes occur simultaneously and jointly reinforce each other. In chapter three, it was shown that IEs expressing a specific PfEMP1 motif can concomitantly bind

EPCR and ICAM-1 and that correlates with a high risk of developing CM. Thus, the additive actions of cytoadhesion of IE to both receptors and a hyperactive immune response may trigger pathways that would promote high expression of ICAM-1 molecules, loss of EPCR expression, increased release of pro-inflammatory cytokines such as TNF-α and endothelial activation (reviewed in Hviid and Jensen, 2015). Increased production of TNF-α mediates the release of other cytokines including HMGB1 that further causes the release of more TNF-α leading to a vicious cycle resulting in the upregulation of ICAM-1 and the further amplification of IE sequestration and associated shedding of soluble forms. Endothelial activation may trigger the exocytosis of Ang-2 from Weibel Palade bodies resulting in increased blood-brain barrier leakage and elevation of the soluble forms of EPCR and ICAM-1 in circulation. Indeed, microvascular obstruction, inflammation, cerebral oedema, blood-brain-barrier dysfunction and breakdown are notable features in paediatric CM (reviewed in Storm and Craig, 2014; Miller *et al*., 2013; Clark *et al*., 2006).

Thus, targeted host response studies combined with analysis of specific PfEMP1 motifs could unravel new mechanisms of protection from cerebral IE sequestration including the development of therapeutic interventions. Overall, this study demonstrates that endothelial activation characterised by high levels of soluble adhesion molecules and inflammatory mediators are observed in Ghanaian children with acute malaria.

CHAPTER SIX

CHARACTERISATION OF BLOOD OUTGROWTH ENDOTHELIAL CELL (BOEC) *IN VITRO* SYSTEM AND ITS POTENTIAL USE TO STUDY THE ADHESION PROPERTIES OF *PLASMODIUM FALCIPARUM*-INFECTED ERYTHROCYTES

6.1 Introduction

The endothelium is made up of a monolayer of endothelial cells lining the vascular system and is capable of interacting with its environment. In its steady state, the vascular endothelium regulates blood homeostasis, suppresses coagulation, and acts as a barrier to molecules within the circulation. By contrast, activation of the endothelium can disrupt all these properties resulting in coagulation and vascular leakage (reviewed in Aird, 2012; Pate *et al*., 2010; Galley and Webster, 2004; Cines *et al*., 1998). In *P. falciparum* infection, the vascular endothelium is an active participant. Significantly, IEs interact with adhesion receptors enriched on the surface of endothelial cells, stimulating the endothelial cells to upregulate the expression of these receptors on their surface in response to the infection. Indeed, cytoadhesion of IEs, mediated by the parasite protein PfEMP1, to host endothelial receptors including ICAM-1 and EPCR is a prominent pathological feature in SM. This adhesion process results in a build-up of sequestered IEs in the microvascular beds of vital organs (Hviid and Jensen, 2015; Storm and Craig, 2014). IE sequestration has been linked to a significant risk of developing CM as previous post-mortem studies reveal high numbers of sequestered IEs in brain vessels of patients who died from CM (Milner, Jr. et al., 2014; Milner, Jr. et al., 2014); (Taylor et al., 2004; Silamut et al., 1999; MacPherson et al., 1985). Sequestration, accompanied by the release of toxic parasite molecules and a distorted host inflammatory cytokine response, lead to marked

increase in expression of adhesion proteins, endothelial dysfunction and a collapse in the vascular barrier integrity (Seydel *et al*., 2015; Cunnington *et al*., 2013b; Miller *et al*., 2013; Dorovini-Zis *et al*., 2011).

Research attempting to unlock the processes involved in parasite cytoadhesion and endothelial dysfunction related to malaria pathology has been largely based on evaluations of autopsy samples and use of animal models (Milner, Jr. *et al*., 2014; Craig *et al*., 2012; Franke-Fayard *et al*., 2010; White *et al*., 2010; Taylor *et al*., 2004). Although these models have offered insightful snapshots into disease mechanisms, they have some challenges. The currently available animal models only partially replicate the pathological features of SM. Notably, sequestration of erythrocytes infected with *P. falciparum* in brain tissues has not been observed. Hence their relevance to disease understanding in humans has been intensely challenged (El-Assaad *et al*., 2014; Franke-Fayard *et al*., 2010; White *et al*., 2010). As a result, the study of parasite sequestration in the brain has been confined to the evaluation of autopsy samples which are carried out at end point. The challenge associated with this particular assessment is that it is impossible to investigate the early phase of clinical symptoms. Additionally, there are ethical and cultural restrictions or barriers in obtaining cerebral tissues for investigations (El-Assaad *et al*., 2014; White, 2011; Taylor *et al*., 2004; Medana *et al*., 2001).

In vitro sequestration systems remain essential for studying the adhesive interactions between IEs and endothelial cells in the absence of suitable animal models. Most models used have been derived from non-human sources such as transfected Chinese hamster ovary cells as well as human sources, which include melanoma cells and primary cells (Rogerson *et al*., 1995; Hasler *et al*., 1993; Swerlick *et al*., 1992; Schmidt *et al*., 1982). Although important knowledge has been gained from these

routinely used *in vitro* cells, they have some limitations (Andrews *et al*., 2005). Significantly, primary cell sources are usually pooled samples obtained from different individuals and thus may not ideally reflect *in vivo* situations in malaria patients while others are known to exhibit a limited life span as well as phenotypic drift. Even more, the processes involved in obtaining some of the physiologically relevant endothelial cells are invasive and time consuming and as such cannot be routinely applied (Wassmer *et al*., 2011). Given the clinical importance of parasite adhesive interactions with the host endothelium, there is a recognised need to develop more tools since that could result in opportunities to guide the development of medicines and adjunctive therapies.

Blood outgrowth endothelial cells [BOECs] (Lin *et al*., 2000), also referred to as endothelial colony forming cells [ECFCs] (Ingram *et al*., 2004) could serve as a suitable alternative cell source to study *falciparum* parasite adhesive interactions. BOECs are a sub-fraction of circulating rare cells (0.010 %) in peripheral mononuclear cells of healthy individuals and have differentiated matured endothelial cell phenotypes (Dauwe *et al*., 2016; Timmermans *et al*., 2007; Yoder *et al*., 2007; Ingram *et al*., 2004). Their levels are thought to be modified in some disease states such as sickle cell anaemia where high levels signify the inflamed status of the endothelium (Sakamoto *et al*., 2013; Strijbos *et al*., 2009; Solovey *et al*., 1997). Also in malaria, significantly raised circulating levels have been implicated in CM complications (Oduro, 2015). These cells can easily be harvested from peripheral blood, adapted to *in vitro* cultures with enhanced expansion capacities while maintaining a stable phenotype and are believed to be well suited for vascular regeneration (Dauwe *et al*., 2016; Martin-Ramirez *et al*., 2012; Yoon *et al*., 2005). The identity of BOECs is confirmed by a combination of characteristics including

their morphological features in cultures, expression of endothelial surface antigens and endothelial specific functional assays (Fuchs *et al*., 2010). They display cobblestone morphology reminiscent of endothelial cells and also express cell surface markers such as CD146, von Willebrand factor and CD31. These cells possess the ability to form capillary-like structures on Matrigel and are able to incorporate acetylated low-density lipoproteins (Ingram *et al*., 2004; Lin *et al*., 2000). BOECs have been successfully utilised to study diseases associated with endothelial perturbation and to investigate differential transcript levels of endothelial genes (Sakamoto *et al*., 2013; Chang *et al*., 2008; Fernandez *et al*., 2005). Thus BOECs derived from malaria patients could potentially be a practical alternative *in vitro* system to evaluate the adhesive properties between *P. falciparum* parasites and the host endothelial cells.

6.2 Hypothesis and specific objectives

The study hypothesized that BOECs can be derived from the peripheral blood of malaria patients

Specific objectives

1. To isolate and culture BOECs from PBMCs obtained from malaria patients
2. To profile the surface expression markers on BOECs

6.3 Methods

6.3.1 Sample collection and processing and isolation of peripheral blood mononuclear cells

Samples for this study were collected during the 2016 malaria transmission season at the Hohoe Municipal Hospital in Ghana (see chapter four section 4.3.1). Briefly, heparinised blood samples (3 mL-8 mL) were obtained from 34 hospitalised children (< 12 years old) after written consent was given and processed for peripheral blood mononuclear cells (PBMCs) within 1 h of collection. Briefly, the blood sample was carefully layered on top of an equal volume of Ficoll-Paque solution (GE Healthcare, Sweden) in a 50 mL conical flask and centrifuged (400 g; 15 min) without brakes. Three different phases were observed after centrifugation. The topmost plasma layer was aspirated. The white blood cells containing the PBMCs formed the middle layer and that was collected into a new 50 mL falcon tube. The bottom layer contained the Ficoll and was discarded. The PBMCs were washed with RPMI 1640 with 25 mM Hepes modified (Sigma-Aldrich, Germany), supplemented with 50 µg/mL gentamicin sulfate (Sigma-Aldrich, Germany), [referred to as incomplete RPMI] and centrifuged (400 *g;* 10 min) with brakes and the wash process repeated again. The cells were frozen in 60 % incomplete RPMI, 30 % foetal bovine serum (FBS) [Sigma-Aldrich, Germany] and 10 % Dimethyl sulfoxide (Sigma-Aldrich, Germany) in a Mr. Frosty freeze container at -80 ^{0}C freezer overnight and then transferred the next day to liquid Nitrogen. The PBMCs were subsequently transported on dry ice to Copenhagen and further stored in a freezer at -150 ^{o}C.

6.3.2 Thawing of PBMCs

PBMCs collected from the field were removed from the -150 ^{o}C freezer on ice to the laboratory. Cells were rapidly thawed at 37 ^{0}C and transferred to 12 mL round bottom tubes containing 4 mL of BOEC medium. The cell suspension was centrifuged (300 g; 5 min), resuspended in 2mL BOEC medium and seeded into 6-well plate precoated with collagen (50 µg/mL in DPBS). The plates were incubated (37 ^{0}C ; 5 % CO_2) and the medium was carefully aspirated to remove non-adherent and cellular debris after 48 h. Thereafter, the medium was changed thrice a week until first passage or discarded at day 30 for cells that did not produce BOECs. Cells were visualised everyday under a Leica inverted microscope (Leitz Wetzlar, Germany) and distinct BOEC colonies were observed between six and 20 days after plating. Cells that generated BOECs covering about 60 % of the surface area of the well were passaged into 25 cm^2 flasks for continuous expansion.

6.3.3 Preparation of rat tail collagen type I-coated tissue culture surfaces

Rat tail collagen type I (BD Biosciences, USA) was diluted in Dulbecco's phosphate-buffered saline (DPBS; Sigma-Aldrich, Germany) to obtain a final concentration of 50 µg/mL, filtered and stored at 4 ^{0}C for one month. A volume of 2 mL of collagen I solution was aliquoted into each well of a 6-well tissue culture plate, 3 mL/25-cm^2 flasks and 6 mL/75-cm^2 flasks. Collagen coated plates or flasks were incubated overnight at 4 ^{0}C or for 2 h at 37 ^{0}C in 5 % CO_2 atmosphere. Before seeding of PBMCs or BOECs, the collagen solution was removed and the coated plates or flasks were washed once with DPBS (2 mL/well for 6-well plates, 3 mL/25-cm^2 flasks, and 6 mL/75-cm^2 flasks).

6.3.4 PBMCs and BOEC cultures

BOECs were generated from thawed PBMCs according to (Martin-Ramirez *et al*., 2012) with slight modifications. PBMCs were seeded in 6-well plates and monitored carefully until BOECs appeared. Both PBMCs and BOEC cultures were maintained at 37 ^{0}C in a humidified incubator under 5 % CO_2 atmosphere and in endothelial cell basal medium (EBM-2) [Lonza, USA] supplemented with 10 % FBS (Sigma-Aldrich, Germany) and a set of SingleQuots^{TM} kit (Lonza, USA). The EBM-2 together with supplements will now be referred to as BOEC medium. The volume of BOEC medium used for maintaining cells were 2 mL/well for 6-well plates, 8 mL/25-cm^2 flasks, and 15 mL/75-cm^2 flasks, unless otherwise stated.

6.3.5 Passage of cells

BOECs were split at 80-90 % confluency, depending on the requirement. The culture medium was aspirated from flask and pre-warmed PBS was added and swirled around to wash attached cells. The PBS was aspirated and the wash process repeated again. To detach cells, 1-2 mL of 0.025 % trypsin-EDTA (Sigma-Aldrich, Germany) was added to the cells and incubated at 37 ^{0}C for 5 min until all the cells were rounded up and lifted from the surface of the flask. The activity of trypsin was stopped by addition of 5-10 mL BOEC medium and the cell suspension transferred to a 12 mL round bottom tube and centrifuged (300 g; 5 min). The supernatant was gently decanted, cells were resuspended in BOEC medium and were usually split 1:4 for continuous expansion of culture in collagen coated 25 cm^2 flasks. The cell suspension was incubated for about 2 h to allow cells to adhere and the medium exchanged to remove non-attached cells with pre-warmed fresh BOEC medium.

6.3.6 Freezing of BOECs

BOECs at about 80 % confluency in a 75 cm^2 flask were trypsinised as detailed above. Cells were then resuspended in a freeze mix comprising 60 % BOEC medium, 30 % FBS (Sigma-Aldrich, Germany) and 10 % Dimethyl sulfoxide (Sigma-Aldrich, Germany) to a concentration of 10^6 cells /mL which were aliquoted (1 mL each) into cryotubes. Cells were stored at -80 ^{0}C freezer overnight in MR Frosty freezing container and subsequently transferred to -150 ^{0}C freezer.

6.3.7 Thawing of BOECs

Frozen stocks (1 mL) were transferred from the -150 ^{0}C freezer on ice, thawed at 37 ^{0}C and transferred to 12 mL round bottom tubes containing 4 mL of BOEC medium. The cell suspension was centrifuged (300 g; 5 min) and the supernatant gently decanted. The cells were resuspended in 8 mL BOEC medium, seeded on a collagen coated 25 cm^2 flask and incubated (37 ^{0}C; 5 % CO_2). The medium was replaced with 8 mL BOEC medium the next day.

6.3.8 Phenotypic assessment of BOECs

Flow cytometry was carried out to phenotypically characterise cultured BOECs using cells between third and sixth passage from eight different donors. Cultures showing greater than 80 % confluence in 75 cm^2 flasks were used for the assay. One flask containing BOEC was stimulated with human recombinant TNF-α (10 ng/mL; Thermofisher Scientific, USA) overnight and another culture flask served as resting cells. At least, three independent experiments in duplicates were carried out for all successfully expanded BOEC cultures. Resting or stimulated BOEC cultures were detached with trypsin-EDTA as described before (section 6.3.5). BOECs were washed

once with PBS, resuspended in cold PBS and viable cells counted using the trypan blue (VWR, USA) exclusion method in a Neubauer chamber. BOECs were centrifuged (300 x g; 5 min) and resuspended in PBS (Thermofisher Scientific, Germany) supplemented with 3 % BSA (Saveen Werner AB, Sweden) [assay buffer] to a concentration of $1x10^6$/mL. Hundred microlitres of resuspended BOECs was aliquoted into wells and incubated (45 min; 4 ^{0}C) in the dark with primary monoclonal mouse antibodies against human CD146 (0.25 µg/mL; Biolegend, Switzerland), human CD31 (0.1 µg/mL; R&D Systems, USA), human ICAM-1 (0.1 µg/mL; R&D Systems, USA), human thrombomodulin (0.1 µg/mL; TM) [R&D Systems, USA], human CD36 (0.1 µg/mL; Beckman-Coulter, Germany) or goat polyclonal antibody against human EPCR (1.5 µg/mL; R&D Systems, USA). Cells were washed three times with assay buffer and incubated (30 min; 4 ^{0}C) with fluorescein isothiocyanate (FITC) conjugated anti-mouse (1:200; Vector Laboratories, USA), FITC conjugated anti-goat secondary IgG (1:200; Vector Laboratories, USA) or FITC conjugated mouse anti-human CD133 (1:10; Miltenyi Biotec, Germany) where appropriate. Following washing, cells were resuspended in assay buffer (100 µL) and data acquired on an FC500 cytometer (Beckman-Coulter, Denmark).

6.3.9 Analysis

Data on patients from which PBMCs were obtained were summarised as mean ± SD. Flow cytometry data were obtained from at least three independent experiments in duplicate wells with different donors and were analysed in Winlist 6.0 (Verity Software House). Unstained cells were used as a negative control and as a gating strategy for determination of stained and positive cells. Histograms of cell number versus logarithmic FITC fluorescence intensity were recorded. The mean fluorescence

intensity (MFI) for each of the tested cell surface antigen was determined and expressed as the geometric mean intensity ± SD. Graphical representations and statistical analysis of data were done in GraphPad Prism 7 software (GraphPad Software Inc., San Diego, USA). Differences between continuous variables were determined using a paired T-test with significance set at $p \leq 0.05$.

6.4 Results

6.4.1 Patients' demographics

The general characteristics of patients enrolled are found in Table 6.1. PBMCs were isolated from blood samples (3 mL-8 mL) obtained from 34 children with malaria (mean age 5.2 $\pm$ 3.0 years) with similar numbers of males and females (Table 6.1). The mean age did not differ significantly between genders.

6.4.2 Success rate of BOEC colony establishment and morphological characterisation

Thawed PBMCs were seeded on pre-coated collagen wells and cultures were examined daily using an inverted light microscope. A heterogeneous population consisting of round shaped cells and also distinct spindle-shaped adherent cells characteristic of early endothelial progenitor-like cells were observed between three and six days (Not shown). Most of these spindle-appearing cells died and detached within two weeks. Prominent cobblestone-like clusters typical of endothelial cells (Figure 6.1A) emerged in some cultures from day 6 to 20. Overall, 41 % (Table 6.1) of the PBMCs seeded generated colonies, while 64.3 % (Table 6.1) of these were observed within eight days after seeding. Colonies appearing in initial cultures after

15 days were observed to have a comparatively low proliferative capacity and could not be maintained beyond passage one. Further, 64.3 % of the generated colonies were observed in children ≤ 5years old. Expanded BOEC colonies readily attached strongly to the collagen extracellular matrix within minutes of seeding, formed confluent monolayers (Figure 6.1A) in culture flasks within five days in 75cm^2 flasks and maintained cell to cell contact. The majority (71.4 %) of observed BOECs successfully reached passage three (minimum passage number for assay; Table 6.1). BOECs pre-treated overnight with human TNF-α greatly altered their morphological appearance as they assumed elongated structures (Figure 6.1B) with no apparent increase in cell numbers when compared with resting BOECs.

Table 6.1: Patients profile and success rate of BOEC colony generation

Number of patients	34
Age in years (mean±SD)	5.2±3.0
Gender (male)	17/34 (50.0 %)
Success rate of colony generation	14/34 (41.2 %)
Success rate in ≤ 5 y	64.3 %
Day of appearance in donor wells	
Within 8 days	9/14 (64.3 %)
After 8 days	5/14 (35.7 %)
Success to passage 3	10/14 (71.4 %)

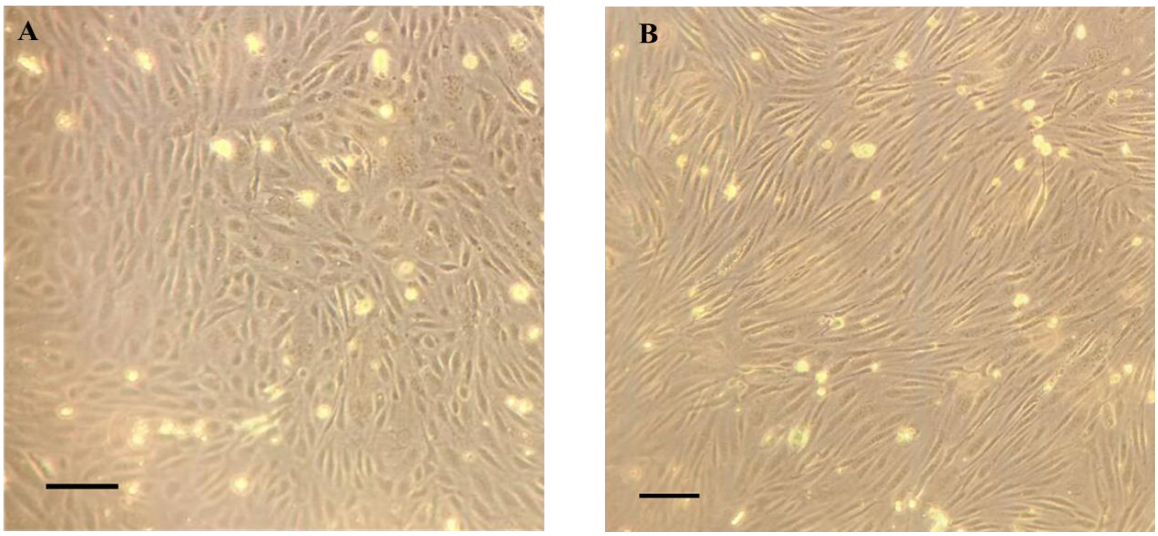


Figure 6.1: Morphology of expanded BOEC cultures in resting (A) and activated (B) states. BOECs readily attached to collagen upon seeding and formed confluent monolayers within three to five days of culturing. **(A).** Resting cells appeared cobblestone-like. **(B)** Overnight activation with TNF-α resulted in elongated cells (representative images). Bright round cells indicate dead floating cells. Bar indicates 100 µm.

6.4.3 Phenotypic assessment of resting and activated BOECs

BOEC cultures were expanded and their surface antigen expression simultaneously assessed in both resting and TNF-α activated states using cells between passages three and six. The phenotype of resting and activated BOECs was assessed with specific antibodies targeting surface exposed markers including a positive endothelial marker CD146 and a negative control stem cell marker CD133 in flow cytometry experiments. Data were shown as MFI of expression of cell surface antigen markers (Figures 6.2A-6.2B, Table 6.2). Results for BOEC resting cultures showed that these cells constitutively expressed endothelial cell surface antigens CD146, CD31 and TM (Figure 6.2A, Table 6.2). In addition, cell surface adhesion molecules including ICAM-1 and EPCR were expressed (Figure 6.2A, Table 6.2). Specifically, high expression levels were recorded for CD146 and CD31, moderate levels were seen in EPCR and TM and very weak surface expression documented for ICAM-1 (Table 6.2). Conversely, BOECs tested negative for the endothelial cell marker CD36 and the immature stem cell marker CD133 (Table 6.2). The inflammatory response of these cells was assessed by overnight treatment with 10 ng/mL of TNF-α and tested in flow cytometry assays (Figure 6.2B, Table 6.2). In comparison with resting cells, TNF-α treatment induced the expression of ICAM-1 significantly (Student's paired t-test; $p<0.001$), while the expression of CD146 and CD31 were marginally increased (Student's paired t-test; $p < 0.05$). By contrast, the expression of cell surface EPCR and TM levels was considerably reduced by overnight activation with TNF-α (Student's paired t-test; $p<0.01$). Pretreatment with TNF-α did not induce the surface expression of CD36 on BOECs (Table 6.2).

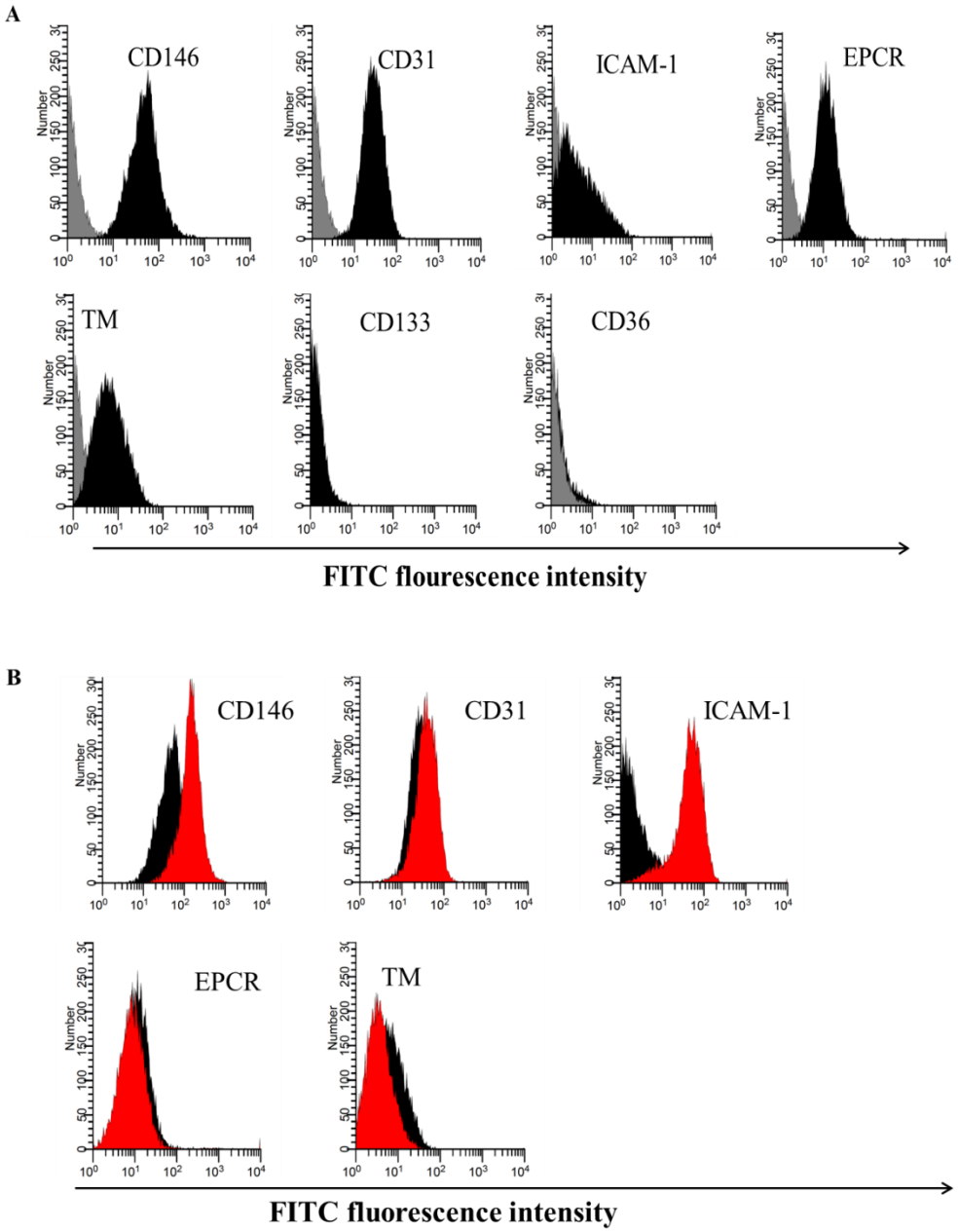


Figure 6.2: Characteristics of cell surface antigens. (A). Phenotypic characterisation of cell surface antigens of resting BOEC cultures assessed by flow cytometry using cells between passage three and six. Representative histograms showing overlays with gray histogram representing negative control and black histograms positively stained for CD146, CD31, ICAM-1, EPCR, TM and negatively stained for CD133 and CD36. **(B)**. Comparative cell surface antigen expression in resting and activated states. BOECs activated overnight with 10 ng/mL of TNF-α showed increased expression of CD146, CD31 and ICAM-1, and decreased EPCR and TM expression. Representative histograms showing overlays with black histogram representing resting cells and red histograms for activated cells stained for CD146, CD31, ICAM-1, EPCR and TM.

Table 6.2: Flow cytometry assessment of BOEC cells

CD	Antigen	Clone	Resting (mean MFI±SD)	Activated (mean MFI±SD)
CD54	ICAM-1	BBIG-11	3.01±2.12	34.87±15.08***
CD36	GPIV	FA6.152	0.19±0.05	0.23±0.08
CD31	PECAM-1	9G11	26.67±31.32	34.80±27.43*
CD141	TM	501733	4.01±1.97	1.67±0.95**
CD133	Prominin 1	AC133	0.002±0.005	0.001±0.007
CD146	MCAM	P1H12	67.83±22.82	105.70±17.42**
CD201	EPCR	Polyclonal	10.96±2.27	7.85±1.40**

Quantitative comparison of BOEC in resting and activated states was done using flow cytometry. Results represent the mean MFI±SD of cells expressing surface antigens from at least three independent experiments with cells propagated from different donors (n=8) and normalised to the mean MFI values of unstained cells. Analysis done by paired Student T-test (***$p < 0.001$; **$p < 0.01$; *$p < 0.05$) CD: cluster of differentiation; ICAM-1: intercellular adhesion molecule 1; GP IV: Glycoprotein four; TM: thrombomodulin; EPCR: endothelial protein C receptor; PECAM-1: Platelet endothelial cell adhesion molecule 1; MCAM: melanoma cell adhesion molecule

6.5 Discussion

The discovery that rare precursors of endothelial progenitor cells resident in PBMCs can be isolated and propagated *in vitro* to yield endothelial cell-like phenotype (Asahara *et al*., 1997) raised significant interest in exploiting their potential therapeutic use in vascular regeneration. Their usefulness as a research tool in studying disorders linked to the vascular endothelium was also explored (Sakamoto *et al*., 2013; Martin-Ramirez *et al*., 2012; Fuchs *et al*., 2010; Fernandez *et al*., 2005). Since this significant finding, several studies have provided evidence that progenitors circulating in peripheral blood contain a heterogeneous population of cells (Hur *et al*., 2004; Gulati *et al*., 2003; Lin *et al*., 2000). Among the diverse phenotypes characterised, is a unique population of cells so called BOEC or ECFC; these cells

display highly similar features to mature and differentiated endothelial cells in terms of their morphological appearance, surface antigen expression profile and the potential to form capillary-like networks on Matrigel (Fuchs *et al*., 2010; Yoder *et al*., 2007; Ingram *et al*., 2004; Lin *et al*., 2000). The present study evaluated the feasibility of isolating and characterising the surface antigens on BOEC from malaria patients as a potentially useful *in vitro* cell model to study malaria parasite adhesion properties. Overall, the study demonstrated for the first time that PBMCs from Ghanaian malaria patients, propagated on the basis of adhesion and maintenance in an endothelial selective culture medium, can yield BOEC colonies. The data further showed that BOECs can be readily established from small blood volumes and from frozen PBMC samples.

Following seeding of thawed isolated PBMCs, spindle-like structures described as early circulating endothelial progenitors (Hur *et al*., 2004; Gulati *et al*., 2003) were observed in cultures between three and six days after culturing and this has been previously documented (Estes *et al*., 2010; Yoder *et al*., 2007; Ingram *et al*., 2004). The appearance of these structures possibly indicated that cells were appropriately frozen and viable. BOEC colonies were successfully observed in approximately 41 % of the PBMCs donors (Table 6.1). Earlier studies have recorded over 70 % success in the establishment of BOEC colonies. It is important to emphasize that these studies (Dauwe *et al*., 2016; Ormiston *et al*., 2015; Tura *et al*., 2013; Ingram *et al*., 2004) used relatively large volumes of blood (40 mL- 100 mL) compared to the volumes used in this study (3 mL-8 mL) and also some studies had similar or lower success rate of isolation in comparison with this study while using large volume (Campioni *et al*., 2013; Medina *et al*., 2013; Sakamoto *et al*., 2013; Mutunga *et al*., 2001). Moreover, most previous studies have generated BOEC from freshly harvested PBMCs whereas

this study shows for the first time, the generation of BOECs from frozen PBMCs. This may have contributed to the relatively low success rate. Furthermore, available data indicate that BOECs cannot be generated from some individuals and the reason for such difficulty remains to be investigated, although increased age has been suggested to be a potential confounder (Medina *et al*., 2013; Martin-Ramirez *et al*., 2012). A greater majority of the BOEC colonies (~64 %) emerged in individual cultures within eight days of seeding (Table 6.1). Previous findings have reported the appearance of colonies characteristic of BOECs from five days onwards post seeding, although most studies typically detect them between 14-21 days (hence referred to as late colony forming) (Bou *et al*., 2015; van Beem *et al*., 2009; Zhang *et al*., 2009; Yoder *et al*., 2007; Ingram *et al*., 2004; Lin *et al*., 2000). Although it is difficult to precisely explain these variations, they could possibly be related to factors such as material source, methods of propagation or genetic differences in study populations. A significant challenge in this field is the differing protocols and culture conditions employed in the derivation of these cells, resulting in high variabilities in individual findings (Gumina and Su, 2017; Fujinaga *et al*., 2016; Glynn and Hinds, 2014). Most studies characterising BOECs have obtained samples from individuals of non–African origin, unlike this study where donors were of African origin.

BOEC cultures were expanded and phenotypically characterised based on their morphological appearance in culture and expression of specific endothelial cell surface antigens (Ingram *et al*., 2004). Characterisation of the cell surface antigens showed that despite donor variations, BOEC abundantly expressed CD146 and CD31 which are characteristics of endothelial cells (Figure 6.2; Table 6.2). They also expressed the anti-thrombogenic antigens TM and EPCR, whereas the proinflammatory inducible marker ICAM-1 was scarcely expressed on their surface

(Table 6.2). These phenotypes observed on resting cells have been repeatedly documented in many studies (Wang *et al*., 2013b; Ahmann *et al*., 2011; Yoder *et al*., 2007; Lin *et al*., 2002; Lin *et al*., 2000). Resting BOECs were negative for the endothelial cell-specific antigen CD36 and the immature stem cell marker CD133 (Figure 6.2; Table 6.2). Few studies have described CD36 expression on some population of BOECs (Sakamoto *et al*., 2013; Lin *et al*., 2000) and in these studies, an enrichment-based surface marker isolation method was employed. Thus, their method of isolation highly differed from this study; moreover, there is evidence showing that isolation and propagation methods employed have a remarkable influence on the type of BOECs generated (Glynn and Hinds, 2014; Delorme *et al*., 2005). In addition, it is well-recognised that there is considerable heterogeneity in the expression of surface antigens and adhesion molecules on endothelial cells from different vascular beds (Aird, 2007; Galley and Webster, 2004). The absence of CD133 expression demonstrated that these cells were derived from non-haematopoietic progenitors (Tura *et al*., 2013; Wang *et al*., 2013b; Timmermans *et al*., 2007). Taken together, the combined surface expression of several antigens gave credence to their endothelial phenotypic identity (Sakamoto *et al*., 2013; Wang *et al*., 2013b; van Beem *et al*., 2009; Ingram *et al*., 2004; Lin *et al*., 2000).

Endothelial cells play diverse roles during inflammation, increasing their expression of adhesion molecules and secreting several cytokines (Galley and Webster, 2004; Cines *et al*., 1998). The present study exposed BOECs to the pro-inflammatory cytokine TNF-α overnight. A profound alteration in the morphology was observed in TNF-α activated cells, notably, they appeared elongated rather than cobblestone-like when observed under the microscope (Figure 6.1). The altered cobblestone morphology has been observed before in human endothelial cells and that was

attributed to the redistribution of surface expression markers like CD31 resulting from TNF-α stimulation (Romer *et al*., 1995). Characterisation of the cell surface antigens of these TNF-α activated cells showed a marked induction in the expression of surface ICAM-1 in contrast to the scarce expression of this adhesion molecule on resting cells (Figure 6.2; Table 6.2). The surface expression levels of CD146 and CD31 were slightly elevated (Figure 6.2; Table 6.2). By contrast, TM and EPCR expression levels were noticeably decreased and no change was observed in the expression levels of CD36 which was negative on resting cells (Figure 6.2; Table 6.2). The marked alteration in the expression of these surface antigens in response to the cytokine TNF-α, documented in this study, are in accordance with many investigations (Sakamoto *et al*., 2013; Ahmann *et al*., 2011; van Beem *et al*., 2009; Zhang *et al*., 2009; Lin *et al*., 2000).

Infection by *P. falciparum* is often characterised by sequestration resulting from cytoadhesion of IEs to endothelial receptors in vital organs (Miller *et al*., 2013). Thus investigations attempting to understand in detail this deadly adhesion process remain critical for discovering therapeutic interventions against severe disease development. However, research into this process has been greatly hampered by the lack of appropriate animal models. The scarcity of autopsy samples with its related ethical concerns has also created an additional level of complication (El-Assaad *et al*., 2014; Taylor *et al*., 2004). Other clinically relevant endothelial cell sources including vascular-derived and adipose-derived microvascular endothelial cells have been investigated, however procedures for obtaining these cells are invasive, which is an undesirable process for the donor and hence not an optimal material source (Wassmer *et al*., 2011; Muanza *et al*., 1996; Swerlick *et al*., 1992). *In vitro* endothelial cell models still remain indispensable in advancing our understanding on cytoadhesion

processes. A model system that naturally expresses the receptors that are known to be involved in malaria pathology can provide details on the processes and also generate hypotheses for further investigations. In *falciparum* cytoadhesion interactions, clinically important parasite receptors on the host endothelium include EPCR and ICAM-1 (Lennartz *et al*., 2017; Turner *et al*., 2013; Turner *et al*., 1994). Indeed, data from chapter three showed that simultaneous adhesion to EPCR and ICAM-1 by IEs was strongly associated with CM complications. The BOECs isolated from this study expressed these molecules on their surface; even more, their surface expression was altered in response to the proinflammatory cytokine TNF-α. This cytokine has often been implicated in malaria pathogenesis; particularly high levels have been recorded in CM patients (Hunt and Grau, 2003; Kwiatkowski *et al*., 1990; Grau *et al*., 1989), thus they could serve as useful *in vitro* model in probing IE sequestration in CM.

An obvious concern for considering BOECs as a study tool is that they are primary cell lines, as such are prone to lose their characteristics with many passages (age), differentiate into a non-endothelial phenotype or become senescent, thus restricting their usage to early passages (Medina *et al*., 2013; Medina *et al*., 2010; van Beem *et al*., 2009; Au *et al*., 2008). However, it is worthwhile to mention that these cells have been shown to exhibit a stable phenotype over many passages (Szoke *et al*., 2016; Medina *et al*., 2010; Fuchs *et al*., 2006; Lin *et al*., 2002). Moreover, it is possible to obtain high yield at low passages which can be stored for future use (Medina *et al*., 2013; Medina *et al*., 2010; Zhang *et al*., 2009; Lin *et al*., 2002). Furthermore, BOECs also have a significant advantage over other primary cells since they can be easily accessed. There is no specific marker(s) that unequivocally identifies mature endothelial cells particularly BOECs, as such, it is recommended that a detailed combination of molecular and functional assessments are carried out. In keeping with

this practice, in future, functional assays including their capacity to form capillary-like networks *in vitro*, internalisation of acetylated low density lipoproteins and pattern of localisation of cell surface antigens would be done to confirm their endothelial phenotype (Medina *et al*., 2013; Yoder *et al*., 2007; Bompais *et al*., 2004).

Although this study did not provide evidence that these cells can support malaria parasite adhesion, preliminary data from our laboratory using BOECs derived from healthy European donors showed that they support the adhesion of erythrocytes infected with *P. falciparum* (Dr. Yvonne Adams, personal communication, University of Copenhagen). Moreover, the expression of putative parasite receptors on these cells suggests their utility and experiments are ongoing to demonstrate their adhesive properties. These cells can be easily and conveniently obtained from any consenting malaria patient since blood is usually taken minimally-invasively for standard clinical management, thus placing no extra burden on the patient. The procedures utilised in the isolation and establishment of BOECs described in this study are simple and can be routinely performed successfully. Notably, PBMCs can be easily isolated in field settings, frozen down, transported and subsequently cultured when convenient without loss of cell viability. Furthermore, these cells are culture adaptable, grow rapidly and can progressively be passaged to obtain large amounts. These remarkable characteristics make them attractive candidates as well as a physiologically appropriate cell and practical alternative *in vitro* parasite sequestration system.

In summary, this study shows that BOECs can be successfully isolated and culture-expanded from paediatric malaria patients and thus offers the feasibility of using patient-derived BOECs to study parasite adhesive interactions and also test therapies aimed at improving endothelial function.

CHAPTER SEVEN

FREQUENCY OF IGM BINDING PFEMP1 VARIANTS IN THE GENOME OF *P. FALCIPARUM*

7.1 Introduction

The mechanisms underpinning SM development remain largely unexplained. However, cytoadhesion of IEs to an array of receptors on the host endothelium, to uninfected erythrocytes in a process known as rosetting, and to two or more IEs to form clumps via platelet binding are well-recognised pathophysiological features (reviewed in Rowe *et al*., 2009). These adhesion processes culminating in the sequestration of the mature forms of IEs in deep microvascular beds is largely responsible for organ-specific disease pathology including CM and PAM (Milner, Jr. *et al*., 2014; Taylor *et al*., 2004; Salanti *et al*., 2003; Fried and Duffy, 1996). The polymorphic high molecular weight adhesive protein family known as PfEMP1 is thought to be responsible for IE sequestration (Howard *et al*., 1988; Leech *et al*., 1984b).

PfEMP1 are composed of variable adhesive domains of Duffy binding-like (DBLα, β, γ, δ, ε, ζ and x) and cysteine-rich interdomain regions [CIDRα, β, γ and δ] (reviewed in Hviid and Jensen, 2015) allowing the parasite to bind numerous host receptors. There is compelling evidence showing that the ability of some IEs expressing PfEMP1 subtypes to mediate adhesion requires support from soluble proteins present in human plasma (Stevenson *et al*., 2015a; Stevenson *et al*., 2015b; Clough *et al*., 1998; Scholander *et al*., 1996). Although several plasma proteins have been implicated, only the pentameric form of non-immune IgM has been extensively described (Stevenson *et al*., 2015a; Barfod *et al*., 2011; Scholander *et al*., 1998). Notably, IEs responsible for rosette formation in children and CSA binding associated with placental malaria

are recognised as having a high affinity for IgM and both phenotypes have profound correlation to development of SM (Creasey *et al*., 2003; Rowe *et al*., 2002). Although the biological consequence of this binding is less clear, it is believed that the pentameric nature of IgM facilitates the grouping of multiple IEs together in order to fortify the adhesive interactions between IEs and their receptors on erythrocytes among rosetting parasites (Akhouri *et al*., 2016; Stevenson *et al*., 2015a). In PAM however, IgM binding appears to be an escape mechanism where targeted epitopes are shielded from immune destruction without jeopardizing IEs binding capacity to CSA (Barfod *et al*., 2011). The FCμ4 portion of the pentameric IgM is believed to interact with DBLζ and DBLε domains of PfEMP1 molecules on the surface of IEs (Stevenson *et al*., 2015a; Ghumra *et al*., 2008; Semblat *et al*., 2006). Altogether, this suggests that the parasite may utilise IgM to promote sequestration and hence avoid immune clearance which is critical to its survival as well as sustaining chronic infections.

Previous studies have consistently reported that some PfEMP1 domains bind non-immune IgM (Jeppesen *et al*., 2015; Stevenson *et al*., 2015a; Ghumra *et al*., 2008; Rasti *et al*., 2006; Semblat *et al*., 2006). Thus, it is of high importance to investigate the number of IgM binders in the parasite genome given their potential connection with severe disease phenotypes. Keeping with this notion, only one study has characterised the number of PfEMP1 proteins in one parasite genome (NF54/3D7) with high binding affinity for IgM and this provided evidence of at least five PfEMP1 variants involved in IgM binding (Jeppesen *et al*., 2015). Of interest, one of the identified variants was found to mediate CSA adhesion (NF54 VAR2CSA), whereas the functional roles of the others could not be elucidated (Jeppesen *et al*., 2015). It is possible that the frequency of PfEMP1 variants involved in IgM binding may have

been grossly underestimated. This study aimed at identifying and characterising additional and new PfEMP1 variants that mediate IgM binding in the NF54/3D7 genome. Such information may increase our understanding of these adhesive processes and hopefully be crucial for designing strategies for interfering with IgM binding, which will reduce parasite sequestration and ultimately allow the immune system to control the infection.

7.2 Hypothesis and specific objectives

In this study, it was hypothesized that there are several IgM binding PfEMP1 molecules in the genome of the *P. falciparum* isolate NF54 which have not yet been characterised

Specific objectives

1. To identify the number of *var* genes upregulated in the NF54 genome following selection for IgM binding
2. To evaluate the IgM binding property of the identified *var* encoding PfEMP1 proteins

7.3 Methods

7.3.1 Parasite culture

The previously generated *P. falciparum* NF54 clone, G6 (Frank *et al*., 2007) was transfected with a plasmid construct pVBH that carried the gene encoding blasticidin S deaminase (BSD) under the control of a UpsC *var* gene promoter. The *P. falciparum* NF54 pVBH-transfected G6 clone was kindly donated by Dr. Ron Dzikowski. The

procedures for generating transfectants have been previously described (Deitsch *et al*., 2001a). Briefly, electroporation cuvettes (0.2 cm) were loaded with 175 µL of uninfected erythrocytes and 50 µg of plasmid DNA in incomplete cytomix solution. Parasite populations that stably maintained the plasmid were first selected on 2 µg/mL blasticidin and thereafter, high copy number transfectants were subsequently generated by culturing in 10 µg/mL blasticidin. For this particular study, the stably transfected NF54 G6 clone was maintained in RPMI 1640 with 25 mM Hepes modified (Sigma-Aldrich, Germany), 50 µg/mL gentamicin sulfate (Sigma-Aldrich, Germany), 4 mM Glutamine (Sigma-Aldrich, Germany), 0.5 % Albumax II (Invitrogen, USA) (referred to as 0.5 % Albumax II medium) and blasticidin (10 µg/mL; Life Technologies, USA) to turn off transcription of endogenous *var* genes and wipe out the epigenetic memory. Cultures released from selective drug pressure were maintained in 0.5 % Albumax II (Invitrogen, USA) only using previously described methods (see chapter three section 3.3.9). The parasites were routinely genotyped (Snounou *et al*., 1999) and absence of mycoplasma contamination was regularly tested using the MycoAlert Mycoplasma Detection Kit (Lonza, USA) according to the manufacturer's protocol.

7.3.2 IE selection and single-cell cloning of IgM-binding IEs

Antibody selection of IEs and single cell cloning were done following previously described procedures (Jeppesen *et al*., 2015). The pVBH-NF54 G6 clone maintained under blasticidin pressure was released from the selection pressure and maintained for two weeks in 0.5 % Albumax II culture medium only. Late stage IEs were purified by MACS as described before. Purified IEs (2 x 10^6 IEs/mL) were incubated (20 min; room temp) with sterile 10 nM human IgM (Sigma-Aldrich, Germany), washed three

times with sterile PBS supplemented with 2 % FBS (2 % FBS) and centrifuged (500 x g 5min; room temp). Then the IEs were stained with sterile phycoerythrin-conjugated donkey anti-human IgM (Jackson Immuno- Research, USA) (1:200; 30 min; room temp) and the wash process repeated (500 x g; 5min; room temp). IgM positive parasites (diluted to 1 x 10^6/mL in 2 % FBS) were then single cell sorted using the fluorescence-activated cell sorter Jazz sorter (Beckton Dickson, Germany) into round-bottom 96-well plates (Thermofisher Scientific, Denmark) containing Albumax II culture medium (100 µL/ well) and uninfected erythrocytes (1 µL/well). Varying numbers of IEs (50-1000) which served as controls were placed in some wells, while the majority of the wells contained single cells. The plates were incubated (37 °C) for two weeks with media replacement carried out thrice weekly with addition of uninfected erythrocytes (1 µL/well) twice a week. Control wells containing high numbers of IEs were used to monitor parasite growth by staining prepared slides with Giemsa (Merck, Germany) and subsequently observing them under the microscope. Control wells were also used for IgM binding in flow cytometry (see section 7.3.5). Thereafter, IgM positive clones were expanded in 25cm^2 culture flasks (Thermofisher Scientific, Denmark).

7.3.3 Repeated selection of IgM positive clones

Late stage IEs were purified by MACS as described before (see chapter three section 3.3.12) and incubated (30 min, room temp) with 10 nM human IgM (Sigma-Aldrich, Germany). Protein A dynabeads (25 µL; Invitrogen, USA) were aliquoted into a 10 mL centrifuge tube and 4 mL of incomplete RPMI was added to wash the beads. The tube containing the beads was placed in a magnetic field; the tube was inverted a few times and then allowed to stand for about 1 min. The wash medium was then aspirated

and the wash process was repeated. IgM (10 nM diluted in incomplete RPMI) was added to the beads and incubated (30 min; room temp) on a rocking table. The tube containing the mixture was placed on a magnet and the wash process repeated thrice to remove unbound IgM. Mature IEs were resuspended in 2 mL of incomplete RPMI, added to the IgM coated beads and incubated for about 30 min on a rocking table. The wash process was repeated thrice to remove unbound parasites. IgM selected parasites were resuspended in 0.5 % Albumax II culture medium (5 mL) at 2 % haematocrit, the mix was transferred to a $25cm^2$ culture flask, gassed and maintained in an incubator (see chapter three section 3.3.9).

7.3.4 TRizol samples and *var* gene transcription analysis

Tightly synchronised ring stage IEs (> 2 %) were collected and preserved in TRizol reagent (Thermofisher Scientific, USA). The *var* transcription profiles were carried out as described in the methods section of chapter three (see chapter three section 3.3.8). Briefly, RNA was extracted from 100 µL ring stage parasites dissolved in 900 µL TRizol reagent (Thermofisher Scientific, USA) and used to generate cDNA (see chapter three section 3.3.7). qPCR was carried out using the synthesized cDNA and *P. falciparum* 3D7 *var* gene-specific primers as described previously (Salanti *et al*., 2003), with the modifications described in (Dahlback *et al*., 2007). Transcript levels relative to the *seryl-tRNA synthetase* housekeeping gene (p90) were determined using the $2^{-\Delta CT}$ method (see chapter three section 3.3.8).

7.3.5 IgM reactivity to the surface of IEs

Growth of IEs and IgM binding were assessed by flow cytometry on a Beckman Coulter FC500 instrument (Beckman Coulter, Denmark) as described before

(Jeppesen *et al*., 2015) using MACS-purified late stage IEs or packed IE aliquots. IEs were incubated (30 min; 4 °C) with IgM (10 nM) and subsequently stained with ethidium bromide (2 µg/mL) and FITC conjugated anti-human IgM (Sigma-Aldrich, Germany; 1:150) for 30 min at 4 °C. Each experiment was done in duplicates.

7.3.6 Data analysis

Calculations to determine the transcription level for each *var* gene relative to the p90 control gene were performed in Microsoft Excel. Comparison of transcript profiles of individual *var* genes within or between different subclonal parasite populations were presented as pie charts. The *var* gene with the highest relative transcription level was assigned as the dominant gene. Analysis of IgM binding data was done using the Winlist 6.0 software (Verity, Topsham).

7.4 Results

7.4.1 *var* transcription profile of NF54 G6 IEs propagated with or without blasticidin

The study made use of the previously derived *P. falciparum* NF54 G6 clone to determine the frequency of PfEMP1 molecules involved in IgM binding. Briefly, the *P. falciparum* NF54-derived G6 clone which almost exclusively transcribes the group C *var* gene PFD1015c was transfected with a blasticidin resistance gene pVBH controlled by a *var* promoter (Fastman *et al*., 2012; Dzikowski and Deitsch, 2008; Frank *et al*., 2007). Transfectants were subsequently selected on 10 µg/mL blasticidin to ensure the carriage of high copy numbers of the plasmids. Maintaining this stably transfected parasite population in 10 µg/mL of blasticidin effectively halts the

transcription of the dominant *var* gene PFD1015c and results in the deletion of the epigenetic memory by the competing episomes, thus selecting for a parasite population that essentially expressed only the BSD gene. In contrast, exclusion of the blasticidin pressure allows the parasite population to begin to shed the pVBH plasmids resulting in the expression of essentially all *var* genes by the parasite population over a period of time in a random fashion (Fastman *et al*., 2012; Dzikowski and Deitsch, 2008).

Taking advantage of this elegant technology, this study evaluated the transcription profile of *var* genes that were overexpressed following the removal of blasticidin pressure from the pVBH-NF54 G6 clone and subsequently selected these parasites for IgM binding. To verify that the BSD selected parasites did not express any *var* genes, RNA extracted from ring stage IEs maintained in blasticidin, were analysed by qPCR using *var* gene specific primers (Dahlback *et al*., 2007; Salanti *et al*., 2003). As expected, the relative levels of all *var* genes including the previous prominently transcribed PFD1005c *var* gene were effectively silenced (Figure 7.1). To investigate the onset of *var* transcription and switching, the blasticidin pressure was removed for two weeks to allow activation of *var* genes in the parasite population. The *var* gene expression profile showed the random transcription of almost all *var* groups at very low levels relative to the housekeeping gene (p90) [Figure 7.1] suggesting the induction of active transcription of endogenous *var* genes in the parasite population in the absence of blasticidin.

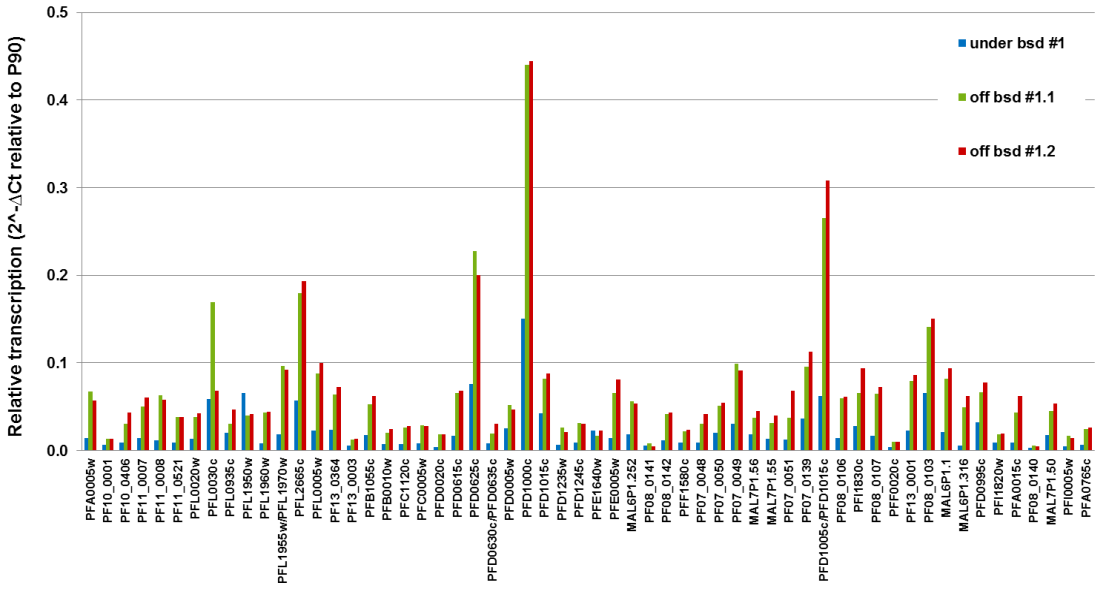


Figure 7.1: *var* gene transcription profile of 3D7/NF54 with or without blasticidin. The relative distribution of each *var* mRNA transcript in the parasite genome is represented as bar charts. Transcript levels were measured by qPCR using a specific set of primers targeting each of the *var* genes present in the 3D7 genome (Salanti *et al*., 2003; Dahlback *et al*., 2007). All values are presented as relative transcript number to the housekeeping gene, *seryl-tRNA synthetase* (p90). Different culturing conditions (under bsd (blue) or off bsd #1.1 (green), 1.2 (red)) are annotated at the topmost right of bar graphs. Off bsd#1.1 (green) and off bsd #1.2 (red) represent two rounds of sorting after release from blasticidin pressure.

7.4.2 IgM selection and *var* gene profile of IgM positive IEs

Following the activation of *var* transcription after drug pressure removal, IEs were stained with IgM and sorted into 96-well plates using fluorescence-activated cell sorting to select for IgM positive cells which were then individually cloned. *In vitro* culturing and expansion resulted in 21 growing cultures arising from the single sorted IEs and one growing culture from a control well that received 1000 sorted IEs (subline). The *var* expression patterns were assayed and the results revealed the transcription of at least nine distinct *var* genes from the IgM positive IEs (Figure 7.2).

All subclonal isolates showed the presence of several sub-population of *var* genes resulting from transcriptional switching, although, dominant transcripts could be

detected in some subclones. The relative transcript levels of some of the *var* gene encoded PfEMP1 implicated in IgM binding in the different subclonal isolates are represented in figure 7.2 (representative images). Notably, eight of the IgM-positive subclonal isolates (G6. 1.14, G6. 5.13, G6. 2.28, G6. 4.80, G6. 3.30, G6. 1.46, G6. 2.72, G6. 3.50) transcribed a particular *var* gene, two transcribed two dominant *var* genes (G6. 3.11, G6. 3.81) at similar levels and the remaining subclones (G6. 3.41, G6. 5.22, G6. 1.90, G6. 3.61, G6. 5.72) and one subline (G6. 3.15) displayed variable expression of multiple *var* genes at very low proportions. With respect to the subtypes of PfEMP1 positive for IgM binding, three subclones solely transcribed PFL0030c (G6 1.14, G6 5.13, G6 2.28), and the following individual subclones: PFD0995c (G6. 3.30), MAL6P1.316 (G6 4.80), PFL2665c (G6 2.72), PF08_0107 (G6 3.50) and PFA0005w (G6 1.46), prominently expressed a unique *var* gene. Also, one subclone showed high transcription for both MAL6P1.1 and PF13_0001 (G6 3.11), whereas another subclone co-expressed PFL2665c and PFL0020w (G6 3.81).

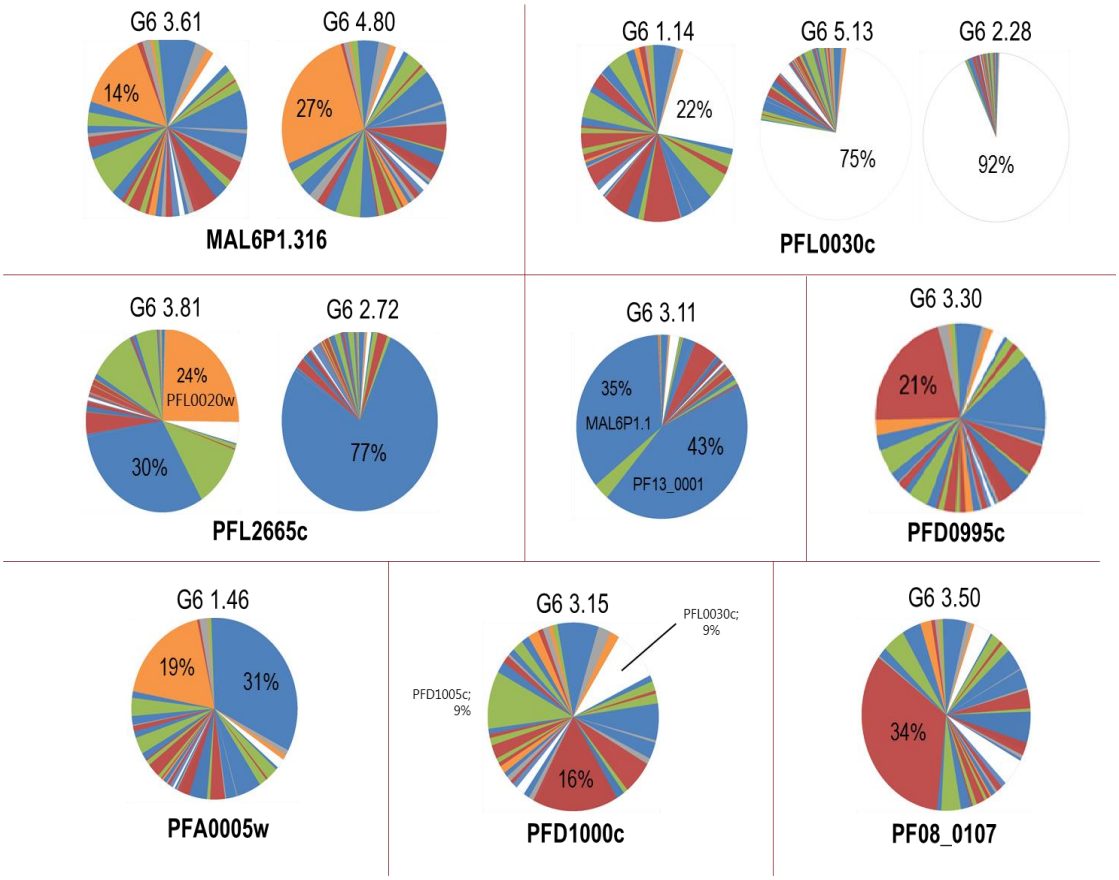


Figure 7.2: *var* gene profiling of IgM positive *P. falciparum* NF54 following subcloning and in vitro expansion. Expression of *var* genes was measured by qPCR and the values presented as relative transcript level to p90. Pies represent the relative distribution of *var* transcripts in the subclonal isolates (identified by a code on the top of each pie chart). Representative images of the major identified transcripts and the corresponding percentage in the various subclonal isolates are indicated on the pie charts. Colour coding and classification of *var* gene groups were assigned as previously described by Fastman *et al*., 2012 and Lavstsen *et al*., 2012 respectively: group A (gray), group B/A (orange), group B (blue), group B/C (green), group C (red) and VAR2CSA white

The domain architecture of the PfEMP1 encoding the dominant *var* genes (Figure 7.3) identified variable numbers of DBLs and CIDRs domains. However, all the PfEMP1 could be placed into two semi-conserved structurally distinct groups. One group which comprised (MAL6P1.316, PFL0020w, PF07_0139 and PFL0030c) contained a similar subtype of DBLε domain with a variable number of this domain in the different *var* gene subtypes (Figure 7.3), which is consistent with a previous finding (Jeppesen *et al*., 2015). Within the DBLε subtype group, two *var* gene types

(MAL6P1.316 and PFL0020w) additionally encoded a DBLζ domain similar to the one described in the HB3VAR06 rosetting parasite isolate which is known to bind IgM (Stevenson *et al*., 2015a). The other semi-conserved group shared a common DBLδ subtype (MAL6P1.1, PFL2665c, PF13_0001, PFD0995c, PF08_0107 and PFA0005w) similar to one of the domains observed in PF07_0139 *var* subtype (Figure 7.3). Thus, the results suggest different PfEMP1 subtypes to be involved in non-immune IgM binding.

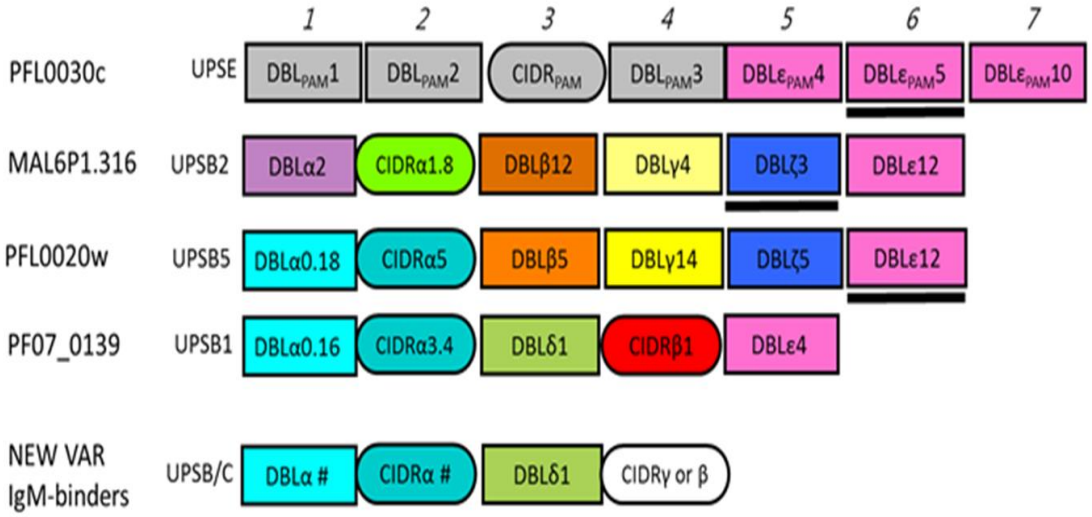


Figure 7.3: Schematic of the extracellular domain organisation of the PfEMP1 proteins encoded by major *var* gene transcripts in identified subclonal IEs. Annotation for the new *var* IgM binders was obtained from the VarDom 1.0 server (http://www.cbs.dtu.dk/services/VarDom/). The domain numbers are indicated in italics along the top of the panel, the names of the PfEMP1 and the *var* promoter groups are indicated at the beginning of each domain structure. The identified IgM binding candidates in this study were PFL0030c, MAL6P1.316, PF07_0139 (Jeppesen *et al*., 2015) and the new *var* encoded PfEMP1 candidates with IgM binding potential (MAL6P1.1, PFL2665c, PF13_0001, PFD0995c, PF08_0107, PFA0005w). The underlined domains have been shown to mediate IgM binding.

7.4.3 Binding of IgM positive PfEMP1 IEs

The ability of IgM positive IEs transcribing some of the identified *var* genes to mediate IgM binding was tested in flow cytometry experiments. The data showed differential levels of IgM binding of the expected *var* genes in the identified subclones (Figure 7.4).

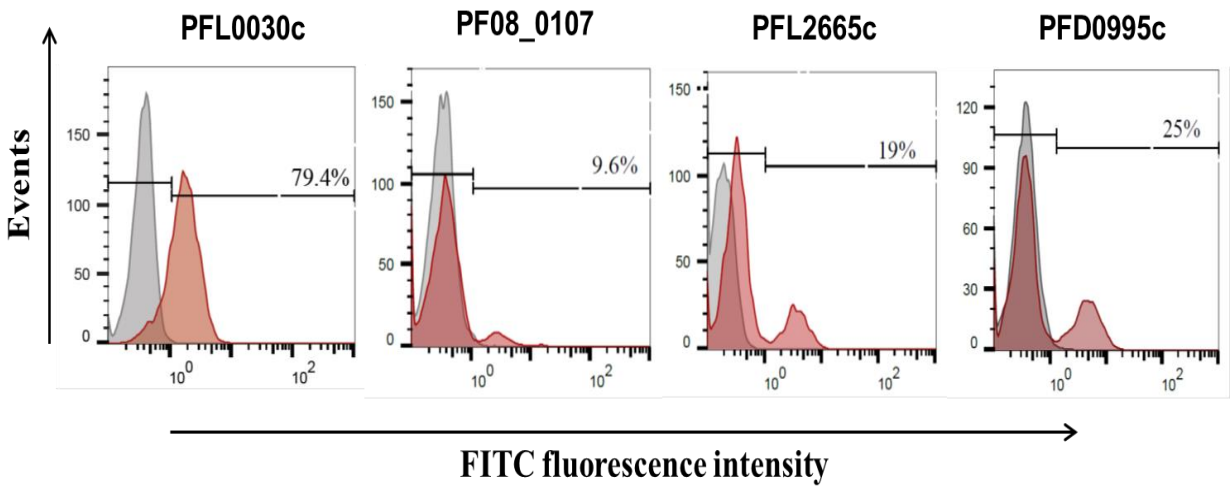


Figure 7.4: IgM binding of IEs expressing PfEMP1. IEs were incubated with 10 nM IgM (30 min; 4 °C) and subsequently stained with 2 µg/mL ethidium bromide and FITC conjugated anti-human IgM (1:150; 30 min; 4 °C) for flow cytometry analysis. Representative histograms showing overlays with gray histogram representing unstained negative control IEs and brown histograms for IEs stained positive for IgM.

7.5 Discussion

Earlier demonstration that rosetting and placental IEs expressing PfEMP1 (Rasti *et al*., 2006; Creasey *et al*., 2003; Clough *et al*., 1998; Scholander *et al*., 1996) share dual binding affinity for the Fc region of IgM has generated considerable interest to unravel the contributions made by IgM to disease severity (Creasey *et al*., 2003; Rowe *et al*., 2002). To date, several PfEMP1 domains have been reported to bind IgM (Jeppesen *et al*., 2015; Stevenson *et al*., 2015a; Ghumra *et al*., 2008; Semblat *et al*., 2006). Recent emerging data revealed that non-CSA binding and non-rosetting PfEMP1 subtypes that bind IgM do exist, further complicating the exact consequence of this interaction (Jeppesen *et al*., 2015). Besides, very little is known about the frequency of these Fc-mediated IgM-binding PfEMP1 phenotypes in the *P. falciparum* genome and it is pertinent to investigate given the potential clinical implications.

This study utilised the pVBH-NF54 G6 transfected parasites in which *var* gene epigenetic mechanism can be reversibly manipulated in a controlled manner. The pVBH-NF54 G6 transfected parasites were initially released of the drug pressure and the transcriptional results showed the expression of nearly all *var* genes (Figure 7.1), an observation which has previously been documented (Jeppesen *et al*., 2015; Fastman *et al*., 2012; Dzikowski and Deitsch, 2008). Following the onset of transcription and the ensuing *var* switching, the parasite population was selected by IgM and positive clones were single sorted and propagated. The *var* transcription profile on expanded IgM positive IEs identified nine different *var* genes among the subclonal isolates as potential candidates for the IgM-binding phenotype (Figure 7.2).

The molecular interactions between PfEMP1 and IgM have been well described. Notably, PfEMP1 domains of the DBLϵ and DBLζ subtypes (interacting with the cμ3

and cμ4 domains of the Fc portion of IgM) have been implicated (Jeppesen *et al*., 2015; Stevenson *et al*., 2015a; Ghumra *et al*., 2008; Rasti *et al*., 2006; Semblat *et al*., 2006). The domain structure of the encoded forms of PfEMP1 identified in this study revealed two semi-conserved structural groups. Members belonging to one group shared a similar DBLε domain structure present at the C-terminal end (PF07_0139, MAL6P1.316, PFL0020w and PFL0030w) with variable representations, whereas the other group had a semi-conserved C-terminal DBLδ domain structure [PFL2665c, PF13_0001, PF08_0107, MAL6P1.1, PFD0995c and PFA0005w] (Figure 7.3).

In the DBLϵ-related domain group is the *var* subtype PFL0030c encoding VAR2CSA in the 3D7 clone that has been strongly connected with pregnancy-associated SM (Salanti *et al*., 2004; Salanti *et al*., 2003). The Fc-mediated IgM binding has been mapped to the C-terminal DBLϵ domains of VAR2CSA expressing IEs (Jeppesen *et al*., 2015; Barfod *et al*., 2011; Rasti *et al*., 2006). The other three non-VAR2CSA *var* gene types with a similar DBLε subtype (MAL6P1.316, PFL0020w, PFL07_0139) discovered in this work were recently identified as IgM binders in a study that characterised IgM binding PfEMP1 in the NF54 parasite (Jeppesen *et al*., 2015). Thus, the results obtained from this study further corroborated their IgM binding identity. Furthermore, two of the identified non-VAR2CSA subtypes (MAL6P1.316, PFL0020w) additionally harboured a DBLζ domain similar to the one found in the well-characterised rosetting parasite isolates TM284VAR1 and HB3VAR06 which are also known to mediate IgM binding (Stevenson *et al*., 2015a; Ghumra *et al*., 2012; Scholander *et al*., 1996). Based on their structural resemblance to HB3VAR06, the ability of MAL6P1.316 and PFL0020w encoding PfEMP1 variants to mediate formation of rosette was investigated in a previous study that first identified them as IgM binding phenotypes. Results showed that these PfEMP1 variants did not mediate

formation of rosettes, indicating that PfEMP1 variants involved in IgM binding were not peculiarly limited to rosette-forming parasites but yet to be discovered phenotypic roles (Jeppesen *et al*., 2015). The results presented in this work indicate that both DBLζ and DBLε at the C–terminal end are important mediators of IgM binding and even more, the data confirmed findings of earlier studies (Jeppesen *et al*., 2015; Stevenson *et al*., 2015a; Ghumra *et al*., 2012).

The other candidates identified in this study had a shared DBLδ structural domain (PFL2665c, PF13_0001, MAL6P1.1, PFD0995c, PF08_0107, PFA0005w) and these have not been previously reported as IgM binding PfEMP1 molecules (Figure 7.3). Furthermore, these putative PfEMP1 molecules did not possess the classical domains that were already known to mediate IgM binding. Notwithstanding, the structurally related C-terminal DBLδ domain found among these members have previously been observed in some well-studied IgM binding PfEMP1 (HB3VAR06, PF07_0139, MAL6P1.4). Thus, it can be speculated that this DBLδ might be an additional domain that can mediate IgM binding.

A detailed characterisation of the newly reported PfEMP1 candidates with potential affinity for IgM was not investigated in this study; as such future studies would have to demonstrate that they are indeed localised on the surface of IEs and that they do mediate IgM binding. Such experiments would involve selecting NF54 IEs with specific antibodies raised against the newly identified PfEMP1 and then subsequently testing the PfEMP1 expressed by the selected IEs for IgM binding as well as assessing the *var* gene transcript profiles of the selected IEs. Furthermore, the exact domains (PfEMP1 and IgM) involved, as well as the affinity of the interactions would have to be determined. Additionally, functional assays (such as rosette formation,

cytoadhesion) to evaluate the potential relevance of IgM binding are needed in order to confirm their phenotypic relevance.

The fact that IgM binding PfEMP1 phenotypes are observed suggest that they may have important roles that promote the survival of the parasite. Indeed, several pathogenic microorganisms have evolved mechanisms that limit their detection by the host immune system, including binding to human immunoglobulins (Lin *et al*., 2004; Medina *et al*., 1999; Bjorck and Kronvall, 1984). It is therefore important that the frequency of IgM binding PfEMP1 in other *falciparum* genomes is characterised in future. Furthermore, other parasite-derived variant surface antigens, including RIFINs which have been implicated in SM complications (Goel *et al*., 2015) need to be investigated to determine if they are co-selected for IgM binding.

Understanding the interaction between the parasite-encoded proteins and IgM would undoubtedly contribute new insights into strategies that can block this adhesion process. In summary, this study was able to identify IgM binders consistent with previous studies. More importantly, we have provided evidence for the existence of several PfEMP1 variants that potentially mediate IgM binding in the genome of the NF54 parasite, that were previously unreported.

CHAPTER EIGHT

GENERAL DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

8.1 General discussion

It is widely acknowledged that malaria-related deaths have markedly reduced globally in the last decade by approximately 40 %. This has been attributed to an increase in control interventions including better testing diagnostics, vector-based interventions and treatment with ACTs (WHO, 2016). Despite such remarkable achievements and intensified efforts, childhood deaths due to *P. falciparum* remain significantly high. Notably, CM, the most deadly form of SM, has a case fatality rate of approximately 20 % (reviewed in Birbeck *et al*., 2010) suggesting that current efforts are still inadequate. Thus, additional measures, including more effective drugs and vaccines will remain paramount for malaria control and a global health priority (reviewed in Crompton *et al*., 2010). Obviously, this can be facilitated by an in-depth understanding of the intricate host-parasite relationship.

The marked sequence diversity found within the PfEMP1 molecules has frustrated efforts in identifying conserved functional regions that could be linked to specific SM syndromes such as CM. Nevertheless, the discovery that VAR2CSA specifically interacts with placental CSA during PAM demonstrates (Salanti *et al*., 2004; Salanti *et al*., 2003) the existence of some conserved PfEMP1 determinants involved in organ-specific severe disease. Although the mechanisms leading to CM complications are only partially understood, cytoadhesion, mediated by the virulent parasite protein PfEMP1 to the ICAM-1 receptor on brain endothelial cells, has been implicated (Bengtsson *et al*., 2013b). Thus, a major objective of this thesis was to dissect the molecular details characterising ICAM-1 binding by group A PfEMP1, by using sequence information to define a sequence motif that can be used to identify ICAM-1-

binding DBLβ domains. Furthermore, it was investigated whether the expression of these DBLβ domains involved in ICAM-1 binding is linked with CM development.

Thus, in chapter three, sequence information and data from structural analysis (from collaborators) of group A DBLβ PfEMP1 proteins binding to ICAM-1 were used to identify a highly conserved sequence motif (the ICAM-1 interaction site). An *in silico* analysis using the motif sequence identified a broad set of motif-containing DBLβ of group A PfEMP1 proteins that mediated ICAM-1 binding. These PfEMP1 proteins also contained an immediate upstream CIDRα domain involved in EPCR binding and thus suggested the ability of these proteins to adhere to both receptors. Indeed, we demonstrated that IEs expressing PfEMP1 containing the motif have a dual binding affinity for the host proteins EPCR and ICAM-1. This binding property allows for increased cytoadhesion and that constitutes an important risk factor for CM development. These findings were further supported by transcriptional data that showed higher transcription of the motif-containing PfEMP1 in CM patients compared with patients with other forms of malaria. The sequences defining the motif were exceptionally conserved, giving an indication that they could trigger cross-reactive responses. Significantly, antibodies from plasma samples of asymptomatic individuals in malaria-endemic regions affinity purified on either the motif-containing DBLβ domain or the motif peptide were able to inhibit ICAM-1 binding of both homologous and heterologous motif-containing DBLβ domains and IEs, indicating that such strain-independent immune responses occur in natural infections and may be involved in acquired protective immunity. Furthermore, purified rat antibodies raised against the motif peptide effectively abolished the interaction of IEs that mediate ICAM-1 binding *in vitro*.

The extreme intra-strain variations found within *var* genes encoding PfEMP1 present a major limitation in considering PfEMP1 as potential vaccines (Trimnell *et al*., 2006). This challenge was highlighted in a previous study that dissected the interaction between PfEMP1 containing CIDRα1 domains and EPCR, a phenotype that has been strongly linked with SM development (Turner *et al*., 2013; Lavstsen *et al*., 2012). A crystal structure of CIDRα1-EPCR complex was used to identify conserved features that could be targeted for therapeutics against SM (Lau *et al*., 2015). The study showed that the overall organisational structure of the CIDRα1 domain of PfEMP1 was preserved to mediate interaction with its receptor, however, the residues that directly interacted with EPCR were highly variable (Lau *et al*., 2015). Thus, the sequence diversity observed may hamper the possibility of generating extensive cross-reactive natural responses against these CDIRα1 residues that directly interact with EPCR. In the present study, using sequence analysis combined with complementary crystal structure data of a group A PfEMP1 binding to ICAM-1 (Lennartz *et al*., 2017) from our collaborators (Professor Matthew Higgins laboratory, Oxford University), we show that the amino acid sequences defining the motif (the residues that directly interact with ICAM-1) were exceptionally conserved. To the best of our knowledge, this is the first report identifying such a conserved motif sequence in a group A PfEMP1 molecule, thus providing a strong rationale for considering the ICAM-1 binding motif as a new target for vaccine development against CM.

In order to make good arguments for vaccine consideration, this study investigated (chapter four) whether plasma antibodies from malaria patients or rat antibodies raised against the motif–containing DBLβ domain or the motif peptide, were cross-reactive and capable of blocking the ICAM-1 interaction with a large selection of DBLβ domain constructs or IEs. The data generated showed that antibodies against motif-

containing DBLβ were acquired in an age-dependent manner. No significant difference in antibody reactivity was detected between SM and UM in motif-containing DBLβ domains. Possible explanations for this observation may be that our sample size was too small to detect any differences. Moreover, CM cases were not detected during the sampling year, partially limiting the analysis that could be done. A plasma pool generated from the enrolled children and affinity purified on the motif-containing DBLβ domain was capable of inhibiting ICAM-1 binding of a heterologous motif-containing DBLβ domain, which further validated our initial observation (chapter three). To show that this inhibition was solely mediated by motif-specific antibodies with biological relevance, rat immunisations were carried out using either motif peptides (the ICAM-1 interaction site) or the motif DBLβ domains. Both the motif-containing DBLβ rat antisera and IgG affinity purified on the motif peptide demonstrated binding inhibition against several heterologous motif-containing ICAM-1-binding DBLβ domains at comparable levels, in addition to interfering with the adhesion of IEs to ICAM-1. More importantly, antisera generated from only two motif-specific peptides were effective at significantly abolishing adhesion of ICAM-1-binding *falciparum* parasites from paediatric SM patients including CM. Altogether, the data demonstrated that broadly reactive cross-inhibitory antibodies against the motif can be acquired following *P. falciparum* natural infections. Additionally, these antibodies could be experimentally induced. Such cross-reactive antibodies interfered with ICAM-1 binding of recombinant motif-containing DBLβ domains and IEs from four out of five paediatric CM patients.

Cross-reactive antibody responses raised against specific PfEMP1 domains or minimal binding regions have been reported before particularly in studies involving VAR2CSA (Bordbar *et al*., 2012; Magistrado *et al*., 2011; Nielsen *et al*., 2009;

Andersen *et al*., 2008), which is the only PfEMP1 based vaccine candidate targeting PAM (Fried and Duffy, 2015). Notably, antibodies generated against the DBL4ε domain demonstrated strain transcending adhesion inhibition activity (Magistrado *et al*., 2011; Nielsen *et al*., 2009). However, antibodies generated against peptides covering epitopes targeted by DBL4ε-specific adhesion blocking antibodies did not recognise the surface of IEs expressing VAR2CSA as well as not mediate blocking of parasite binding to CSA (Ditlev *et al*., 2012). Thus, till now, no single VAR2CSA epitope that could serve as ideal vaccine target has been conclusively identified. By contrast, this study identified a novel conserved linear motif which is the ICAM-1 interaction epitope. Taken together, the results indicate the feasibility of producing an epitope focussed vaccine which aims at eliciting protective immune responses specifically towards the motif.

With the global reduction in malaria deaths as well as the rapid fall in malaria transmission intensity, there have been documented reports of redistribution of malaria cases from younger children to older ones (reviewed in Nkumama *et al*., 2017; Griffin *et al*., 2014). For example, reports from a Gambian study showed a dramatic decline in the number of malaria-related admissions between 2003 and 2007. This was further supported by a significant shift in the mean age of children admitted for malaria from 3.9 years to 5.6 years (Ceesay *et al*., 2008). This observation may possibly be linked to a slower pace in the build-up of protective immunity, although the factors contributing to that remain unclear. A decline in malaria transmission may lead to a reduction in the total mortality which is in line with efforts to eliminate and hopefully eradicate malaria (reviewed in Nkumama *et al*., 2017). Paradoxically, this may increase the risk of resurgent malaria with severe consequences due to the emergence of a population of individuals with little or non-existent naturally-acquired

protective immunity. An effective vaccine against malaria remains the best long-term cost effective and preventive measure. RTS,S is currently the most advanced malaria vaccine (RTS,S, 2015), however, it is well acknowledged that the protection offered by this vaccine is inadequate. This reinforces the need for continued search for efficacious vaccines to accelerate malaria control and eradication efforts. Data from this study raise the optimism of using the motif peptide (the ICAM-1 interaction site) to produce a stand-alone vaccine construct to prevent CM or as part of a multi-component vaccine strategy against SM.

Some additional experiments would have to be done in order to validate the biological importance of the identified ICAM-1 binding motif. Future work using the same experimental procedure described here could be carried out using plasma samples and parasite isolates from patients living in different geographical locations. Notably in Asia, where adult CM is very common and disease phenotypes differ remarkably from paediatric CM. Additionally, prospective longitudinal studies in naturally exposed population are needed to determine the frequency of antibody responses to the ICAM-1 binding motif. This information would be crucial for establishing the involvement of the anti-motif antibodies in the development of immunity and could also serve as a useful marker in sero-epidemiological studies. Furthermore, monoclonal antibodies raised against the motif can be generated and tested for its adhesion blocking activity against IEs.

It is known that SM pathology is complex comprising both parasite cytoadhesion and host inflammatory processes; with both processes occurring concurrently and reinforcing each other (reviewed in Storm and Craig, 2014; Cunnington *et al*., 2013b). In this study, markers of inflammation and endothelial activation (sICAM-1, sEPCR, Ang-2, HMGB1) were investigated in children with SM and UM and also their levels

following resolution of infection (Chapter five). Overall, the data showed higer levels of these soluble markers in malaria patients in comparison with healthy controls. These levels declined significantly in convalescent samples except for HMGB1 which remained high to a similar degree as observed during the infection phase. To the best of our knowledge, this is the first report of raised levels of HMGB1 in convalescent malaria patients which is similar to what has been previously observed in sepsis patients (Sunden-Cullberg *et al*., 2005). Since plasma HMGB1 was measured at only two time points, this study cannot rule out if these levels decline after that. Then, it would be interesting if future studies could determine when these levels decline and whether these elevated levels in convalescent samples have important implications for malaria pathogenesis in endemic populations where multiple infections or reinfections are very common. Overall, the study did not detect differences in the levels of soluble markers between SM and UM, though a trend of higher levels in severe cases was observed. The non-significant differences between both malaria groups could be attributed to our study population. Prior investigations have shown that levels of soluble markers of endothelial activation are highest in CM patients (Lovegrove *et al*., 2009). This study was unable to include enough CM cases possibly due to the rapidly changing malaria epidemiology of our study site where only five CM cases have been recruited in the past three years. Notwithstanding, the data indicate that endothelial activation is a central component of malaria pathogenesis.

Although a highly conserved motif with vaccine potential was discovered (Chapter three), it cannot be considered the magic bullet. There is a consensus that a holistic approach is needed in order to totally eradicate malaria. Thus in future, targeting important immune responses combined with our newly discovered PfEMP1 motif

could unravel new mechanisms of protection from cerebral IE sequestration including the development of therapeutic interventions.

In the absence of suitable animal models (Craig *et al*., 2012; White *et al*., 2010), *in vitro* endothelial cell models still remain fundamental in understanding malaria cytoadhesion processes. Most studies have utilised several cell sources of non-human origin, cancer derived cells from humans or cells from pooled donors usually of non-African origin (Rogerson *et al*., 1995; Hasler *et al*., 1993; Swerlick *et al*., 1992; Dorovini-Zis *et al*., 1991; Schmidt *et al*., 1982). Most of these cells may not reflect the vascular beds of malaria-exposed individuals, and moreover, the procedures in obtaining some of them are time consuming and invasive. This study proposed BOECs from malaria-exposed indivduals as suitable practical cell source to study *falciparum* parasite adhesive interactions. BOECs form part of the circulating rare cell population in peripheral blood with matured endothelial cell phenotypes (Ingram *et al*., 2004; Lin *et al*., 2000). Recently, high circulating levels of these cells have been implicated in CM complications in Ghanaian children (Oduro, 2015). During this study, PBMCs were purified from small volumes of blood obtained from malaria patients to generate BOECs and the surface receptors expressed by these cells were characterised (Chapter six). These cells were shown to express several endothelial markers including ICAM-1 and EPCR and their surface expression was altered in response to the pro-inflammatory cytokine TNF-α.

Of interest, this study provided evidence that the simultaneous adhesion to EPCR and ICAM-1 by IEs is strongly associated with CM complications (Chapter three). Future studies using BOECs could advance our understanding of cerebral cytoadhesion involving both receptors and also serve as a model for assessing potential adjunctive therapies. Indeed data from our laboratory using BOEC derived from European donors

demonstrated that they supported malaria parasite cytoadhesion (Dr. Yvonne Adams, University of Copenhagen, personal communication). Although, prior investigations have isolated and characterised BOECs from healthy individuals as well as individuals with inflammatory vascular disorders (Martin-Ramirez *et al*., 2014; Sakamoto *et al*., 2013; Ingram *et al*., 2004), this study was the first to show that BOECs can be isolated from malaria patients, readily established from small blood volumes and frozen PBMC samples and adapted to *in vitro* culture. Studies are still ongoing to further characterise these cells. Furthermore, malaria patient-derived BOECs would be used to study parasite ligands and the endothelial cell receptors as well as changes in receptor level expression during parasite-adhesive interactions. This offers the possiblility of using endothelial cells derived from malaria-exposed donors of suitable ages and geographic locations and thus provide better physiologically relevant insights into cytoadhesion processes. Consistent with that, a similar approach reported here, could be used to establish BOECs from other geographic regions where SM in non-immune adults is very common. The receptor expression levels of the BOECs generated, including responses to inflammatory cytokines and parasite adhesion could be investigated. The results of these measurements between paediatric malaria and adult malaria patients as well as healthy individuals could also be compared. Such studies may increase our understanding of malaria pathogenesis and may hopefully lead to the identification of novel interventions.

Finally, this study also investigated other PfEMP1 phenotypes that have been implicated in severe disease. Previous findings demonstrated that rosetting and placental IEs expressing PfEMP1 share dual binding affinity for the Fc region of non-immune IgM, both phenotypes associated with severe disease (Barfod *et al*., 2011; Rowe *et al*., 2002). However, very little is known about the frequency of these Fc-

mediated IgM-binding PfEMP1 phenotypes in the *P. falciparum* genome and it is important to investigate given the potential clinical implications. Here, at least six new *var*/PfEMP1 candidates that can potentially mediate binding to IgM in the 3D7 genome are reported, suggesting that PfEMP1-specific IgM binders may have some relevance to the parasite survival (Chapter seven).

This study however, was unable to provide evidence that these new variants are indeed localised on the surface of IE. Additionally, no detailed functional characterisation was carried out due to time constraints. Notwithstanding, there is ongoing work to further investigate these observations and additionally determine the frequency of these IgM binders in the other *falciparum* genomes. Such information will be vital for understanding disease mechanisms and may result in better disease management.

This study has highlighted the contributions made by both host and parasite factors to SM pathogenesis. Detailed understanding of these important factors remains paramount for effective management of malaria. Of interest, this study identified a novel epitope targeted by protective antibodies that can be used to develop a broadly reactive vaccine against CM.

8.2 Conclusions

1. A highly conserved motif was identified using sequence information and that was used to predict a broad set of motif-containing DBLβ of group A PfEMP1 that mediated ICAM-1 binding. IEs expressing PfEMP1 containing the motif have dual binding affinity for the host proteins EPCR and ICAM-1. This

binding property allows for increased cytoadhesion and that constitutes an important risk factor for CM development.

2. Cross-reactive antibody responses against an array of group A PfEMP1 with the motif-containing ICAM-1 recombinant proteins were acquired following natural infections by *P. falciparum* parasites or could be experimentally induced. Such cross-reactive antibodies interfered with ICAM-1 binding of recombinant motif-containing DBLβ domains and IEs from paediatric CM patients.
3. Endothelial activation, characterised by high levels of sICAM-1, sEPCR, plasma Ang-2 and plasma HMGB1 were observed in children with acute malaria.
4. BOECs derived from malaria patients may potentially serve as an easily accessible and physiologically relevant cell source for the study of *falciparum* parasite-adhesive interactions
5. New *var*/PfEMP1 candidates that can potentially mediate binding to IgM were identified in the 3D7/NF54 genome.

8.3 Recommendations

This study identified a PfEMP1 motif involved in CM. Prospective longitudinal studies in naturally exposed population are needed to determine the frequency of motif *var* transcripts and motif-specific antibody responses and correlate these observations with disease outcomes. Additional experiments are highly recommended to optimize motif peptides to induce more broadly neutralising antibody responses.

High levels of markers of endothelial activation were observed in children with acute malaria. Future investigations that concurrently look at markers of endothelial activation and the newly identified PfEMP1 motif could provide useful insights into SM pathogenesis.

The procedures used in this study to characterise patient-derived BOECs can be used in future to isolate BOECs from different categories of malaria patients. These cells can be used to investigate malaria parasite adhesive interactions with the endothelium and responsiveness to cytokines and thus allow comparative analysis of endothelial activation among different individuals and also between clinical categories. Such information may offer clues on why some individuals succumb to life-threatening SM as well as provide new treatment options.

This study identified new *var*/PfEMP1 candidates that can potentially mediate binding to IgM. It is necessary that future studies provide proof that these PfEMP1 variants are indeed expressed in NF54 selected parasites, localised on the surface of IEs and also determine their functionality.

REFERENCES

Abbott,N.J., Ronnback,L., and Hansson,E. (2006). Astrocyte-endothelial interactions at the blood-brain barrier. Nat. Rev. Neurosci. *7*, 41-53.

Abdi,A.I., Fegan,G., Muthui,M., Kiragu,E., Musyoki,J.N., Opiyo,M., Marsh,K., Warimwe,G.M., and Bull,P.C. (2014). *Plasmodium falciparum* antigenic variation: relationships between widespread endothelial activation, parasite PfEMP1 expression and SM. BMC. Infect. Dis. *14*, 170.

Adams,S., Turner,G.D., Nash,G.B., Micklem,K., Newbold,C.I., and Craig,A.G. (2000). Differential binding of clonal variants of *Plasmodium falciparum* to allelic forms of intracellular adhesion molecule 1 determined by flow adhesion assay. Infect. Immun. *68*, 264-269.

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

Adukpo,S., Kusi,K.A., Ofori,M.F., Tetteh,J.K., Amoako-Sakyi,D., Goka,B.Q., Adjei,G.O., Edoh,D.A., Akanmori,B.D., Gyan,B.A., and Dodoo,D. (2013). High plasma levels of soluble intercellular adhesion molecule (ICAM)-1 are associated with cerebral malaria. PLoS. One. *8*, e84181.

Ahmann,K.A., Johnson,S.L., Hebbel,R.P., and Tranquillo,R.T. (2011). Shear stress responses of adult blood outgrowth endothelial cells seeded on bioartificial tissue. Tissue Eng Part A *17*, 2511-2521.

Aikawa,M., Iseki,M., Barnwell,J.W., Taylor,D., Oo,M.M., and Howard,R.J. (1990). The pathology of human cerebral malaria. Am. J. Trop. Med. Hyg *43*, 30-37.

Aird,W.C. (2007). Phenotypic heterogeneity of the endothelium: I. Structure, function, and mechanisms. Circ. Res. *100*, 158-173.

Aird,W.C. (2012). Endothelial cell heterogeneity. Cold Spring Harb. Perspect. Med. *2*, a006429.

Aird,W.C., Mosnier,L.O., and Fairhurst,R.M. (2014). *Plasmodium falciparum* picks (on) EPCR. Blood *123*, 163-167.

Akhouri,R.R., Goel,S., Furusho,H., Skoglund,U., and Wahlgren,M. (2016). Architecture of human IgM in complex with *P. falciparum* Erythrocyte Membrane Protein 1. Cell Rep. *14*, 723-736.

Aley,S.B., Sherwood,J.A., and Howard,R.J. (1984). Knob-positive and knob-negative *Plasmodium falciparum* differ in expression of a strain-specific malarial antigen on the surface of infected erythrocytes. J. Exp. Med. *160*, 1585-1590.

Alleva,L.M., Yang,H., Tracey,K.J., and Clark,I.A. (2005). High mobility group box 1 (HMGB1) protein: possible amplification signal in the pathogenesis of falciparum malaria. Trans. R. Soc. Trop. Med. Hyg *99*, 171-174.

Almelli,T., Nuel,G., Bischoff,E., Aubouy,A., Elati,M., Wang,C.W., Dillies,M.A., Coppee,J.Y., Ayissi,G.N., Basco,L.K., Rogier,C., Ndam,N.T., Deloron,P., and Tahar,R. (2014). Differences in gene transcriptomic pattern of *Plasmodium falciparum* in children with cerebral malaria and asymptomatic carriers. PLoS. One. *9*, e114401.

Alonso,P.L. and Tanner,M. (2013). Public health challenges and prospects for malaria control and elimination. Nat. Med. *19*, 150-155.

Amanna,I.J., Carlson,N.E., and Slifka,M.K. (2007). Duration of humoral immunity to common viral and vaccine antigens. N. Engl. J. Med. *357*, 1903-1915.

Amino,R., Thiberge,S., Martin,B., Celli,S., Shorte,S., Frischknecht,F., and Menard,R. (2006). Quantitative imaging of Plasmodium transmission from mosquito to mammal. Nat. Med. *12*, 220-224.

Amit-Avraham,I., Pozner,G., Eshar,S., Fastman,Y., Kolevzon,N., Yavin,E., and Dzikowski,R. (2015). Antisense long noncoding RNAs regulate *var* gene activation in the malaria parasite *Plasmodium falciparum*. Proc. Natl. Acad. Sci. U. S. A *112*, E982-E991.

Amodu,O.K., Gbadegesin,R.A., Ralph,S.A., Adeyemo,A.A., Brenchley,P.E., Ayoola,O.O., Orimadegun,A.E., Akinsola,A.K., Olumese,P.E., and Omotade,O.O. (2005). *Plasmodium falciparum* malaria in south-west Nigerian children: is the polymorphism of ICAM-1 and E-selectin genes contributing to the clinical severity of malaria? Acta Trop. *95*, 248-255.

Andersen,P., Nielsen,M.A., Resende,M., Rask,T.S., Dahlback,M., Theander,T., Lund,O., and Salanti,A. (2008). Structural insight into epitopes in the pregnancy-associated malaria protein VAR2CSA. PLoS. Pathog. *4*, e42.

Andersson,U. and Tracey,K.J. (2011). HMGB1 is a therapeutic target for sterile inflammation and infection. Annu. Rev. Immunol. *29*, 139-162.

Andersson,U., Wang,H., Palmblad,K., Aveberger,A.C., Bloom,O., Erlandsson-Harris,H., Janson,A., Kokkola,R., Zhang,M., Yang,H., and Tracey,K.J. (2000). High mobility group 1 protein (HMG-1) stimulates proinflammatory cytokine synthesis in human monocytes. J. Exp. Med. *192*, 565-570.

Andrews,K.T., Adams,Y., Viebig,N.K., Lanzer,M., and Schwartz-Albiez,R. (2005). Adherence of *Plasmodium falciparum* infected erythrocytes to CHO-745 cells and inhibition of binding by protein A in the presence of human serum. Int. J. Parasitol. *35*, 1127-1134.

Angeletti,D., Kiwuwa,M.S., Byarugaba,J., Kironde,F., and Wahlgren,M. (2013). Elevated levels of high-mobility group box-1 (HMGB1) in patients with severe or uncomplicated *Plasmodium falciparum* malaria. Am. J. Trop. Med. Hyg *88*, 733-735.

Angus,D.C., Yang,L., Kong,L., Kellum,J.A., Delude,R.L., Tracey,K.J., and Weissfeld,L. (2007). Circulating high-mobility group box 1 (HMGB1) concentrations are elevated in both uncomplicated pneumonia and pneumonia with severe sepsis. Crit Care Med. *35*, 1061-1067.

Anstey,N.M., Russell,B., Yeo,T.W., and Price,R.N. (2009). The pathophysiology of vivax malaria. Trends Parasitol. *25*, 220-227.

Antinori,S., Galimberti,L., Milazzo,L., and Corbellino,M. (2012). Biology of human malaria plasmodia including *Plasmodium knowlesi*. Mediterr. J. Hematol. Infect. Dis. *4*, e2012013.

Armah,H., Wiredu,E.K., Dodoo,A.K., Adjei,A.A., Tettey,Y., and Gyasi,R. (2005). Cytokines and adhesion molecules expressed in the brain in human cerebral malaria. Int. J. Environ. Res. Public Health *2*, 123-131.

Asahara,T., Murohara,T., Sullivan,A., Silver,M., van der Zee,R., Li,T., Witzenbichler,B., Schatteman,G., and Isner,J.M. (1997). Isolation of putative progenitor endothelial cells for angiogenesis. Science *275*, 964-967.

Au,P., Daheron,L.M., Duda,D.G., Cohen,K.S., Tyrrell,J.A., Lanning,R.M., Fukumura,D., Scadden,D.T., and Jain,R.K. (2008). Differential *in vivo* potential of endothelial progenitor cells from human umbilical cord blood and adult peripheral blood to form functional long-lasting vessels. Blood *111*, 1302-1305.

Autino,B., Noris,A., Russo,R., and Castelli,F. (2012). Epidemiology of malaria in endemic areas. Mediterr. J. Hematol. Infect. Dis. *4*, e2012060.

Avraham,I., Schreier,J., and Dzikowski,R. (2012). Insulator-like pairing elements regulate silencing and mutually exclusive expression in the malaria parasite *Plasmodium falciparum*. Proc. Natl. Acad. Sci. U. S. A *109*, E3678-E3686.

Avril,M., Bernabeu,M., Benjamin,M., Brazier,A.J., and Smith,J.D. (2016). Interaction between endothelial protein C receptor and intercellular adhesion molecule 1 to mediate binding of *Plasmodium falciparum*-Infected erythrocytes to endothelial Cells. MBio. *7*.

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., and 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*, E1782-E1790.

Awah,N.W., Troye-Blomberg,M., Berzins,K., and Gysin,J. (2009). Mechanisms of malarial anaemia: potential involvement of the Plasmodium falciparum low molecular weight rhoptry-associated proteins. Acta Trop. *112*, 295-302.

Bachmann,A., Predehl,S., May,J., Harder,S., Burchard,G.D., Gilberger,T.W., Tannich,E., and Bruchhaus,I. (2011). Highly co-ordinated *var* gene expression and switching in clinical *Plasmodium falciparum* isolates from non-immune malaria patients. Cell Microbiol. *13*, 1397-1409.

Baird,J.K. (2013). Evidence and implications of mortality associated with acute Plasmodium vivax malaria. Clin. Microbiol. Rev. *26*, 36-57.

Barber,B.E., William,T., Grigg,M.J., Parameswaran,U., Piera,K.A., Price,R.N., Yeo,T.W., and Anstey,N.M. (2015). Parasite biomass-related inflammation, endothelial activation, microvascular dysfunction and disease severity in vivax malaria. PLoS. Pathog. *11*, e1004558.

Barfod,L., Dalgaard,M.B., Pleman,S.T., Ofori,M.F., Pleass,R.J., and Hviid,L. (2011). Evasion of immunity to *Plasmodium falciparum* malaria by IgM masking of protective IgG epitopes in infected erythrocyte surface-exposed PfEMP1. Proc. Natl. Acad. Sci. U. S. A *108*, 12485-12490.

Barry,A.E., Leliwa-Sytek,A., Tavul,L., Imrie,H., Migot-Nabias,F., Brown,S.M., McVean,G.A., and Day,K.P. (2007). Population genomics of the immune evasion (*var*) genes of *Plasmodium falciparum*. PLoS. Pathog. *3*, e34.

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

Baruch,D.I., Pasloske,B.L., Singh,H.B., Bi,X., Ma,X.C., Feldman,M., Taraschi,T.F., and Howard,R.J. (1995). Cloning the *P. falciparum* gene encoding PfEMP1, a malarial variant antigen and adherence receptor on the surface of parasitized human erythrocytes. Cell *82*, 77-87.

Beare,N.A., Lewallen,S., Taylor,T.E., and Molyneux,M.E. (2011). Redefining cerebral malaria by including malaria retinopathy. Future. Microbiol. *6*, 349-355.

Becker,J.C., Dummer,R., Hartmann,A.A., Burg,G., and Schmidt,R.E. (1991). Shedding of ICAM-1 from human melanoma cell lines induced by IFN-gamma and tumor necrosis factor-alpha. Functional consequences on cell-mediated cytotoxicity. J. Immunol. *147*, 4398-4401.

Bejon,P., Williams,T.N., Nyundo,C., Hay,S.I., Benz,D., Gething,P.W., Otiende,M., Peshu,J., Bashraheil,M., Greenhouse,B., Bousema,T., Bauni,E., Marsh,K., Smith,D.L., and Borrmann,S. (2014). A micro-epidemiological analysis of febrile malaria in Coastal Kenya showing hotspots within hotspots. Elife. *3*, e02130.

Bellamy,R., Kwiatkowski,D., and Hill,A.V. (1998). Absence of an association between intercellular adhesion molecule 1, complement receptor 1 and interleukin 1 receptor antagonist gene polymorphisms and severe malaria in a West African population. Trans. R. Soc. Trop. Med. Hyg *92*, 312-316.

Bengtsson,A., Joergensen,L., Barbati,Z.R., Craig,A., Hviid,L., and Jensen,A.T. (2013a). Transfected HEK293 cells expressing functional recombinant intercellular adhesion molecule 1 (ICAM-1)--a receptor associated with severe *Plasmodium falciparum* malaria. PLoS. One. *8*, e69999.

Bengtsson,A., Joergensen,L., Rask,T.S., Olsen,R.W., Andersen,M.A., Turner,L., Theander,T.G., Hviid,L., Higgins,M.K., Craig,A., Brown,A., and Jensen,A.T. (2013b). A novel domain cassette identifies *Plasmodium falciparum* PfEMP1 proteins binding ICAM-1 and is a target of cross-reactive, adhesion-inhibitory antibodies. J. Immunol. *190*, 240-249.

Berendt,A.R., Ferguson,D.J., Gardner,J., Turner,G., Rowe,A., McCormick,C., Roberts,D., Craig,A., Pinches,R., Elford,B.C., and . (1994). Molecular mechanisms of sequestration in malaria. Parasitology *108 Suppl*, S19-S28.

Berendt,A.R., McDowall,A., Craig,A.G., Bates,P.A., Sternberg,M.J., Marsh,K., Newbold,C.I., and 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*, 71-81.

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

Bergmark,B., Bergmark,R., Beaudrap,P.D., Boum,Y., Mwanga-Amumpaire,J., Carroll,R., and Zapol,W. (2012). Inhaled nitric oxide and cerebral malaria: basis of a strategy for buying time for pharmacotherapy. Pediatr. Infect. Dis. J. *31*, e250-e254.

Bernabeu,M., Danziger,S.A., Avril,M., Vaz,M., Babar,P.H., Brazier,A.J., Herricks,T., Maki,J.N., Pereira,L., Mascarenhas,A., Gomes,E., Chery,L., Aitchison,J.D., Rathod,P.K., and Smith,J.D. (2016). Severe adult malaria is associated with specific PfEMP1 adhesion types and high parasite biomass. Proc. Natl. Acad. Sci. U. S. A *113*, E3270-E3279.

Bernabeu,M. and Smith,J.D. (2017). EPCR and malaria severity: The center of a perfect storm. Trends Parasitol. *33*, 295-308.

Bertin,G.I., Lavstsen,T., Guillonneau,F., Doritchamou,J., Wang,C.W., Jespersen,J.S., Ezimegnon,S., Fievet,N., Alao,M.J., Lalya,F., Massougbodji,A., Ndam,N.T., Theander,T.G., and Deloron,P. (2013). Expression of the domain cassette 8 *Plasmodium falciparum* erythrocyte membrane protein 1 is associated with cerebral malaria in Benin. PLoS. One. *8*, e68368.

Bhasin,V.K. and Trager,W. (1984). Gametocyte-forming and non-gametocyte-forming clones of *Plasmodium falciparum*. Am. J. Trop. Med. Hyg *33*, 534-537.

Bhatt,S., Weiss,D.J., Cameron,E., Bisanzio,D., Mappin,B., Dalrymple,U., Battle,K.E., Moyes,C.L., Henry,A., Eckhoff,P.A., Wenger,E.A., Briet,O., Penny,M.A., Smith,T.A., Bennett,A., Yukich,J., Eisele,T.P., Griffin,J.T., Fergus,C.A., Lynch,M., Lindgren,F., Cohen,J.M., Murray,C.L., Smith,D.L., Hay,S.I., Cibulskis,R.E., and Gething,P.W. (2015). The effect of malaria control on *Plasmodium falciparum* in Africa between 2000 and 2015. Nature *526*, 207-211.

Bhutta,Z.A., Sommerfeld,J., Lassi,Z.S., Salam,R.A., and Das,J.K. (2014). Global burden, distribution, and interventions for infectious diseases of poverty. Infect. Dis. Poverty. *3*, 21.

Birbeck,G.L., Molyneux,M.E., Kaplan,P.W., Seydel,K.B., Chimalizeni,Y.F., Kawaza,K., and Taylor,T.E. (2010). Blantyre Malaria Project Epilepsy Study (BMPES) of neurological outcomes in retinopathy-positive paediatric cerebral malaria survivors: a prospective cohort study. Lancet Neurol. *9*, 1173-1181.

Bjorck,L. and Kronvall,G. (1984). Purification and some properties of streptococcal protein G, a novel IgG-binding reagent. J. Immunol. *133*, 969-974.

Blomqvist,K., Normark,J., Nilsson,D., Ribacke,U., Orikiriza,J., Trillkott,P., Byarugaba,J., Egwang,T.G., Kironde,F., Andersson,B., and Wahlgren,M. (2010). *var* gene transcription dynamics in *Plasmodium falciparum* patient isolates. Mol. Biochem. Parasitol. *170*, 74-83.

Bompais,H., Chagraoui,J., Canron,X., Crisan,M., Liu,X.H., Anjo,A., Tolla-Le,P.C., Leboeuf,M., Charbord,P., Bikfalvi,A., and Uzan,G. (2004). Human endothelial cells derived from circulating progenitors display specific functional properties compared with mature vessel wall endothelial cells. Blood *103*, 2577-2584.

Bordbar,B., Tuikue-Ndam,N., Bigey,P., Doritchamou,J., Scherman,D., and Deloron,P. (2012). Identification of Id1-DBL2X of VAR2CSA as a key domain inducing highly inhibitory and cross-reactive antibodies. Vaccine *30*, 1343-1348.

Bou,K.L., Bouchereau,O., Boulahya,R., Hachem,A., Zaid,Y., Abou-Saleh,H., and Merhi,Y. (2015). Early outgrowth cells versus endothelial colony forming cells functions in platelet aggregation. J. Transl. Med. *13*, 353.

Brown,A., Turner,L., Christoffersen,S., Andrews,K.A., Szestak,T., Zhao,Y., Larsen,S., Craig,A.G., and Higgins,M.K. (2013). Molecular architecture of a complex between an adhesion protein from the malaria parasite and intracellular adhesion molecule 1. J. Biol. Chem. *288*, 5992-6003.

Bull,P.C. and Abdi,A.I. (2016). The role of PfEMP1 as targets of naturally acquired immunity to childhood malaria: prospects for a vaccine. Parasitology *143*, 171-186.

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

Bull,P.C., Buckee,C.O., Kyes,S., Kortok,M.M., Thathy,V., Guyah,B., Stoute,J.A., Newbold,C.I., and Marsh,K. (2008). *Plasmodium falciparum* antigenic variation. Mapping mosaic var gene sequences onto a network of shared, highly polymorphic sequence blocks. Mol. Microbiol. *68*, 1519-1534.

Bull,P.C., Kortok,M., Kai,O., Ndungu,F., Ross,A., Lowe,B.S., Newbold,C.I., and Marsh,K. (2000). *Plasmodium falciparum*-infected erythrocytes: agglutination by diverse Kenyan plasma is associated with severe disease and young host age. J. Infect. Dis. *182*, 252-259.

Bull,P.C., Lowe,B.S., Kortok,M., Molyneux,C.S., Newbold,C.I., and Marsh,K. (1998). Parasite antigens on the infected red cell surface are targets for naturally acquired immunity to malaria. Nat. Med. *4*, 358-360.

Cabrales,P., Zanini,G.M., Meays,D., Frangos,J.A., and Carvalho,L.J. (2010). Murine cerebral malaria is associated with a vasospasm-like microcirculatory dysfunction, and survival upon rescue treatment is markedly increased by nimodipine. Am. J. Pathol. *176*, 1306-1315.

Cabrera,A., Neculai,D., and Kain,K.C. (2014). CD36 and malaria: friends or foes? A decade of data provides some answers. Trends Parasitol. *30*, 436-444.

Calderwood,M.S., Gannoun-Zaki,L., Wellems,T.E., and Deitsch,K.W. (2003). *Plasmodium falciparum var* genes are regulated by two regions with separate promoters, one upstream of the coding region and a second within the intron. J. Biol. Chem. *278*, 34125-34132.

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,L.L., Beld,M.G., Teo,Y.Y., Rockett,K.A., Richardson,A., Kwiatkowski,D.P., Molyneux,M.E., and van Hensbroek,M.B. (2008). Severe anemia in Malawian children. N. Engl. J. Med. *358*, 888-899.

Campioni,D., Zauli,G., Gambetti,S., Campo,G., Cuneo,A., Ferrari,R., and Secchiero,P. (2013). *In vitro* characterization of circulating endothelial progenitor cells isolated from patients with acute coronary syndrome. PLoS. One. *8*, e56377.

Carlson,J., Helmby,H., Hill,A.V., Brewster,D., Greenwood,B.M., and Wahlgren,M. (1990). Human cerebral malaria: association with erythrocyte rosetting and lack of anti-rosetting antibodies. Lancet *336*, 1457-1460.

Ceesay,S.J., Casals-Pascual,C., Erskine,J., Anya,S.E., Duah,N.O., Fulford,A.J., Sesay,S.S., Abubakar,I., Dunyo,S., Sey,O., Palmer,A., Fofana,M., Corrah,T., Bojang,K.A., Whittle,H.C., Greenwood,B.M., and Conway,D.J. (2008). Changes in malaria indices between 1999 and 2007 in The Gambia: a retrospective analysis. Lancet *372*, 1545-1554.

Chakravorty,S.J. and Craig,A. (2005). The role of ICAM-1 in *Plasmodium falciparum* cytoadherence. Eur. J. Cell Biol. *84*, 15-27.

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

Cham,G.K., Turner,L., Lusingu,J., Vestergaard,L., Mmbando,B.P., Kurtis,J.D., Jensen,A.T., Salanti,A., Lavstsen,T., and Theander,T.G. (2009). Sequential, ordered acquisition of antibodies to *Plasmodium falciparum* erythrocyte membrane protein 1 domains. J. Immunol. *183*, 3356-3363.

Chan,J.A., Fowkes,F.J., and Beeson,J.G. (2014). Surface antigens of *Plasmodium falciparum*-infected erythrocytes as immune targets and malaria vaccine candidates. Cell Mol. Life Sci. *71*, 3633-3657.

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

Chang,M.L., Wei,P., Enenstein,J., Jiang,A., Hillery,C.A., Scott,J.P., Nelson,S.C., Bodempudi,V., Topper,J.N., Yang,R.B., Hirsch,B., Pan,W., and Hebbel,R.P. (2008). Genetic endothelial systems biology of sickle stroke risk. Blood *111*, 3872-3879.

Chen,D.S., Barry,A.E., Leliwa-Sytek,A., Smith,T.A., Peterson,I., Brown,S.M., Migot-Nabias,F., Deloron,P., Kortok,M.M., Marsh,K., Daily,J.P., Ndiaye,D., Sarr,O., Mboup,S., and Day,K.P. (2011). A molecular epidemiological study of *var* gene diversity to characterize the reservoir of *Plasmodium falciparum* in humans in Africa. PLoS. One. *6*, e16629.

Chen,Q., Fernandez,V., Sundstrom,A., Schlichtherle,M., Datta,S., Hagblom,P., and Wahlgren,M. (1998). Developmental selection of *var* gene expression in *Plasmodium falciparum*. Nature *394*, 392-395.

Cholewinski,M., Derda,M., and Hadas,E. (2015). Parasitic diseases in humans transmitted by vectors. Ann. Parasitol. *61*, 137-157.

Chookajorn,T., Dzikowski,R., Frank,M., Li,F., Jiwani,A.Z., Hartl,D.L., and Deitsch,K.W. (2007). Epigenetic memory at malaria virulence genes. Proc. Natl. Acad. Sci. U. S. A *104*, 899-902.

Cines,D.B., Pollak,E.S., Buck,C.A., Loscalzo,J., Zimmerman,G.A., McEver,R.P., Pober,J.S., Wick,T.M., Konkle,B.A., Schwartz,B.S., Barnathan,E.S., McCrae,K.R., Hug,B.A., Schmidt,A.M., and Stern,D.M. (1998). Endothelial cells in physiology and in the pathophysiology of vascular disorders. Blood *91*, 3527-3561.

Claessens,A., Adams,Y., Ghumra,A., Lindergard,G., Buchan,C.C., Andisi,C., Bull,P.C., Mok,S., Gupta,A.P., Wang,C.W., Turner,L., Arman,M., Raza,A., Bozdech,Z., and Rowe,J.A. (2012). A subset of group A-like *var* genes encodes the malaria parasite ligands for binding to human brain endothelial cells. Proc. Natl. Acad. Sci. U. S. A *109*, E1772-E1781.

Clark,I.A., Budd,A.C., Alleva,L.M., and Cowden,W.B. (2006). Human malarial disease: a consequence of inflammatory cytokine release. Malar. J. *5*, 85.

Clark,I.A. and Cowden,W.B. (2003). The pathophysiology of falciparum malaria. Pharmacol. Ther. *99*, 221-260.

Clark,I.A. and Rockett,K.A. (1994). Sequestration, cytokines, and malaria pathology. Int. J. Parasitol. *24*, 165-166.

Clark,I.A. and Schofield,L. (2000). Pathogenesis of malaria. Parasitol. Today *16*, 451-454.

Clough,B., Atilola,F.A., Black,J., and Pasvol,G. (1998). *Plasmodium falciparum*: the importance of IgM in the rosetting of parasite-infected erythrocytes. Exp. Parasitol. *89*, 129-132.

Cohen,S., McGregor,I.A., and Carrington,S. (1961). Gamma-globulin and acquired immunity to human malaria. Nature *192*, 733-737.

Coleman,B.I., Ribacke,U., Manary,M., Bei,A.K., Winzeler,E.A., Wirth,D.F., and Duraisingh,M.T. (2012). Nuclear repositioning precedes promoter accessibility and is linked to the switching frequency of a *Plasmodium falciparum* invasion gene. Cell Host. Microbe *12*, 739-750.

Conroy,A.L., Glover,S.J., Hawkes,M., Erdman,L.K., Seydel,K.B., Taylor,T.E., Molyneux,M.E., and Kain,K.C. (2012). Angiopoietin-2 levels are associated with

retinopathy and predict mortality in Malawian children with cerebral malaria: a retrospective case-control study*. Crit Care Med. *40*, 952-959.

Conroy,A.L., Lafferty,E.I., Lovegrove,F.E., Krudsood,S., Tangpukdee,N., Liles,W.C., and Kain,K.C. (2009). Whole blood angiopoietin-1 and -2 levels discriminate cerebral and severe (non-cerebral) malaria from uncomplicated malaria. Malar. J. *8*, 295.

Conroy,A.L., Phiri,H., Hawkes,M., Glover,S., Mallewa,M., Seydel,K.B., Taylor,T.E., Molyneux,M.E., and Kain,K.C. (2010). Endothelium-based biomarkers are associated with cerebral malaria in Malawian children: a retrospective case-control study. PLoS. One. *5*, e15291.

Conroy,A.L., Silver,K.L., Zhong,K., Rennie,M., Ward,P., Sarma,J.V., Molyneux,M.E., Sled,J., Fletcher,J.F., Rogerson,S., and Kain,K.C. (2013). Complement activation and the resulting placental vascular insufficiency drives fetal growth restriction associated with placental malaria. Cell Host. Microbe *13*, 215-226.

Cooke,B.M., Berendt,A.R., Craig,A.G., MacGregor,J., Newbold,C.I., and Nash,G.B. (1994). Rolling and stationary cytoadhesion of red blood cells parasitized by *Plasmodium falciparum*: separate roles for ICAM-1, CD36 and thrombospondin. Br. J. Haematol. *87*, 162-170.

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

Cowman,A.F., Healer,J., Marapana,D., and Marsh,K. (2016). Malaria: biology and disease. Cell *167*, 610-624.

Craig,A., Fernandez-Reyes,D., Mesri,M., McDowall,A., Altieri,D.C., Hogg,N., and Newbold,C. (2000). A functional analysis of a natural variant of intercellular adhesion molecule-1 (ICAM-1Kilifi). Hum. Mol. Genet. *9*, 525-530.

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

Cranmer,S.L., Magowan,C., Liang,J., Coppel,R.L., and Cooke,B.M. (1997). An alternative to serum for cultivation of *Plasmodium falciparum in vitro*. Trans. R. Soc. Trop. Med. Hyg *91*, 363-365.

Crawley,J., Chu,C., Mtove,G., and Nosten,F. (2010). Malaria in children. Lancet *375*, 1468-1481.

Creasey,A.M., Staalsoe,T., Raza,A., Arnot,D.E., and Rowe,J.A. (2003). Nonspecific immunoglobulin M binding and chondroitin sulfate A binding are linked phenotypes of *Plasmodium falciparum* isolates implicated in malaria during pregnancy. Infect. Immun. *71*, 4767-4771.

Crompton,P.D., Moebius,J., Portugal,S., Waisberg,M., Hart,G., Garver,L.S., Miller,L.H., Barillas-Mury,C., and Pierce,S.K. (2014). Malaria immunity in man and mosquito: insights into unsolved mysteries of a deadly infectious disease. Annu. Rev. Immunol. *32*, 157-187.

Crompton,P.D., Pierce,S.K., and Miller,L.H. (2010). Advances and challenges in malaria vaccine development. J. Clin. Invest *120*, 4168-4178.

Cserti-Gazdewich,C.M., Dzik,W.H., Erdman,L., Ssewanyana,I., Dhabangi,A., Musoke,C., and Kain,K.C. (2010). Combined measurement of soluble and cellular ICAM-1 among children with *Plasmodium falciparum* malaria in Uganda. Malar. J. *9*, 233.

Cunnington,A.J., Riley,E.M., and Walther,M. (2013a). Stuck in a rut? Reconsidering the role of parasite sequestration in severe malaria syndromes. Trends Parasitol. *29*, 585-592.

Cunnington,A.J., Walther,M., and Riley,E.M. (2013b). Piecing together the puzzle of severe malaria. Sci. Transl. Med. *5*, 211ps18.

Czajkowsky,D.M., Salanti,A., Ditlev,S.B., Shao,Z., Ghumra,A., Rowe,J.A., and Pleass,R.J. (2010). IgM, Fc mu Rs, and malarial immune evasion. J. Immunol. *184*, 4597-4603.

Dahlback,M., Lavstsen,T., Salanti,A., Hviid,L., Arnot,D.E., Theander,T.G., and Nielsen,M.A. (2007). Changes in *var* gene mRNA levels during erythrocytic development in two phenotypically distinct *Plasmodium falciparum* parasites. Malar. J. *6*, 78.

Dauwe,D., Pelacho,B., Wibowo,A., Walravens,A.S., Verdonck,K., Gillijns,H., Caluwe,E., Pokreisz,P., van,G.N., Carmeliet,G., Depypere,M., Maes,F., Vanden Driessche,N., Droogne,W., Van,C.J., Vanhaecke,J., Prosper,F., Verfaillie,C., Luttun,A., and Janssens,S. (2016). Neovascularization potential of blood outgrowth endothelial cells from patients with stable ischemic heart failure is preserved. J. Am. Heart Assoc. *5*, e002288.

Day,N.P., Hien,T.T., Schollaardt,T., Loc,P.P., Chuong,L.V., Chau,T.T., Mai,N.T., Phu,N.H., Sinh,D.X., White,N.J., and Ho,M. (1999). The prognostic and pathophysiologic role of pro- and antiinflammatory cytokines in severe malaria. J. Infect. Dis. *180*, 1288-1297.

de Jong,G.M., Slager,J.J., Verbon,A., van Hellemond,J.J., and van Genderen,P.J. (2016). Systematic review of the role of angiopoietin-1 and angiopoietin-2 in *Plasmodium* species infections: biomarkers or therapeutic targets? Malar. J. *15*, 581.

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

de Souza,J.B. and Riley,E.M. (2002). Cerebral malaria: the contribution of studies in animal models to our understanding of immunopathogenesis. Microbes. Infect. *4*, 291-300.

Deitsch,K., Driskill,C., and Wellems,T. (2001a). Transformation of malaria parasites by the spontaneous uptake and expression of DNA from human erythrocytes. Nucleic Acids Res. *29*, 850-853.

Deitsch,K.W., Calderwood,M.S., and Wellems,T.E. (2001b). Malaria. Cooperative silencing elements in *var* genes. Nature *412*, 875-876.

Delorme,B., Basire,A., Gentile,C., Sabatier,F., Monsonis,F., Desouches,C., Blot-Chabaud,M., Uzan,G., Sampol,J., and Dignat-George,F. (2005). Presence of endothelial progenitor cells, distinct from mature endothelial cells, within human CD146+ blood cells. Thromb. Haemost. *94*, 1270-1279.

Deloron,P., Dumont,N., Nyongabo,T., Aubry,P., Astagneau,P., Ndarugirire,F., Menetrier-Caux,C., Burdin,N., Brelivet,J.C., and Peyron,F. (1994). Immunologic and biochemical alterations in severe falciparum malaria: relation to neurological symptoms and outcome. Clin. Infect. Dis. *19*, 480-485.

Diamond,M.S., Staunton,D.E., de Fougerolles,A.R., Stacker,S.A., Garcia-Aguilar,J., Hibbs,M.L., and Springer,T.A. (1990). ICAM-1 (CD54): a counter-receptor for Mac-1 (CD11b/CD18). J. Cell Biol. *111*, 3129-3139.

Ditlev,S.B., Nielsen,M.A., Resende,M., Agerbaek,M.O., Pinto,V.V., Andersen,P.H., Magistrado,P., Lusingu,J., Dahlback,M., Theander,T.G., and Salanti,A. (2012). Identification and characterization of B-cell epitopes in the DBL4epsilon domain of VAR2CSA. PLoS. One. *7*, e43663.

Dodoo,D., Omer,F.M., Todd,J., Akanmori,B.D., Koram,K.A., and Riley,E.M. (2002). Absolute levels and ratios of proinflammatory and anti-inflammatory cytokine production *in vitro* predict clinical immunity to *Plasmodium falciparum* malaria. J. Infect. Dis. *185*, 971-979.

Dodoo,D., Staalsoe,T., Giha,H., Kurtzhals,J.A., Akanmori,B.D., Koram,K., Dunyo,S., Nkrumah,F.K., Hviid,L., and Theander,T.G. (2001). Antibodies to variant antigens on the surfaces of infected erythrocytes are associated with protection from malaria in Ghanaian children. Infect. Immun. *69*, 3713-3718.

Dodoo,D., Theisen,M., Kurtzhals,J.A., Akanmori,B.D., Koram,K.A., Jepsen,S., Nkrumah,F.K., Theander,T.G., and Hviid,L. (2000). Naturally acquired antibodies to

the glutamate-rich protein are associated with protection against *Plasmodium falciparum* malaria. J. Infect. Dis. *181*, 1202-1205.

Dondorp,A.M. (2008). Clinical significance of sequestration in adults with severe malaria. Transfus. Clin. Biol. *15*, 56-57.

Dondorp,A.M., Kager,P.A., Vreeken,J., and White,N.J. (2000). Abnormal blood flow and red blood cell deformability in severe malaria. Parasitol. Today *16*, 228-232.

Dondorp,A.M., Nosten,F., Yi,P., Das,D., Phyo,A.P., Tarning,J., Lwin,K.M., Ariey,F., Hanpithakpong,W., Lee,S.J., Ringwald,P., Silamut,K., Imwong,M., Chotivanich,K., Lim,P., Herdman,T., An,S.S., Yeung,S., Singhasivanon,P., Day,N.P., Lindegardh,N., Socheat,D., and White,N.J. (2009). Artemisinin resistance in Plasmodium falciparum malaria. N. Engl. J. Med. *361*, 455-467.

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

Dorovini-Zis,K., Schmidt,K., Huynh,H., Fu,W., Whitten,R.O., Milner,D., Kamiza,S., Molyneux,M., and Taylor,T.E. (2011). The neuropathology of fatal cerebral malaria in malawian children. Am. J. Pathol. *178*, 2146-2158.

Duah,N.O., Matrevi,S.A., Quashie,N.B., Abuaku,B., and Koram,K.A. (2016). Genetic diversity of *Plasmodium falciparum* isolates from uncomplicated malaria cases in Ghana over a decade. Parasit. Vectors. *9*, 416.

Duffy,M.F., Noviyanti,R., Tsuboi,T., Feng,Z.P., Trianty,L., Sebayang,B.F., Takashima,E., Sumardy,F., Lampah,D.A., Turner,L., Lavstsen,T., Fowkes,F.J., Siba,P., Rogerson,S.J., Theander,T.G., Marfurt,J., Price,R.N., Anstey,N.M., Brown,G.V., and Papenfuss,A.T. (2016). Differences in PfEMP1s recognized by antibodies from patients with uncomplicated or severe malaria. Malar. J. *15*, 258.

Duraisingh,M.T., Voss,T.S., Marty,A.J., Duffy,M.F., Good,R.T., Thompson,J.K., Freitas-Junior,L.H., Scherf,A., Crabb,B.S., and Cowman,A.F. (2005). Heterochromatin silencing and locus repositioning linked to regulation of virulence genes in *Plasmodium falciparum*. Cell *121*, 13-24.

Dzikowski,R. and Deitsch,K.W. (2008). Active transcription is required for maintenance of epigenetic memory in the malaria parasite *Plasmodium falciparum*. J. Mol. Biol. *382*, 288-297.

Dzikowski,R., Frank,M., and Deitsch,K. (2006). Mutually exclusive expression of virulence genes by malaria parasites is regulated independently of antigen production. PLoS. Pathog. *2*, e22.

Edgar,R.C. (2004). MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Res. *32*, 1792-1797.

El-Assaad,F., Combes,V., and Grau,G.E. (2014). Experimental models of microvascular immunopathology: The example of cerebral malaria. J. Neuroinfect. Dis. *5*.

Elhassan,I.M., Hviid,L., Satti,G., Akerstrom,B., Jakobsen,P.H., Jensen,J.B., and Theander,T.G. (1994). Evidence of endothelial inflammation, T cell activation, and T cell reallocation in uncomplicated *Plasmodium falciparum* malaria. Am. J. Trop. Med. Hyg *51*, 372-379.

Epp,C., Li,F., Howitt,C.A., Chookajorn,T., and Deitsch,K.W. (2009). Chromatin associated sense and antisense noncoding RNAs are transcribed from the *var* gene family of virulence genes of the malaria parasite *Plasmodium falciparum*. RNA. *15*, 116-127.

Esser,C., Bachmann,A., Kuhn,D., Schuldt,K., Forster,B., Thiel,M., May,J., Koch-Nolte,F., Yanez-Mo,M., Sanchez-Madrid,F., Schinkel,A.H., Jalkanen,S., Craig,A.G., Bruchhaus,I., and Horstmann,R.D. (2014). Evidence of promiscuous endothelial binding by *Plasmodium falciparum*-infected erythrocytes. Cell Microbiol. *16*, 701-708.

Estes,M.L., Mund,J.A., Ingram,D.A., and Case,J. (2010). Identification of endothelial cells and progenitor cell subsets in human peripheral blood. Curr. Protoc. Cytom. *Chapter 9*, Unit-11.

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

Febbraio,M., Hajjar,D.P., and Silverstein,R.L. (2001). CD36: a class B scavenger receptor involved in angiogenesis, atherosclerosis, inflammation, and lipid metabolism. J. Clin. Invest *108*, 785-791.

Fernandez,L., Sanz-Rodriguez,F., Zarrabeitia,R., Perez-Molino,A., Hebbel,R.P., Nguyen,J., Bernabeu,C., and Botella,L.M. (2005). Blood outgrowth endothelial cells from Hereditary Haemorrhagic Telangiectasia patients reveal abnormalities compatible with vascular lesions. Cardiovasc. Res. *68*, 235-248.

Fernandez-Reyes,D., Craig,A.G., Kyes,S.A., Peshu,N., Snow,R.W., Berendt,A.R., Marsh,K., and Newbold,C.I. (1997). A high frequency African coding polymorphism in the N-terminal domain of ICAM-1 predisposing to cerebral malaria in Kenya. Hum. Mol. Genet. *6*, 1357-1360.

Ferreira,M.U., Zilversmit,M., and Wunderlic,G. (2007). Origins and evolution of antigenic diversity in malaria parasites. Curr. Mol. Med. *7*, 588-602.

Fiedler,U. and Augustin,H.G. (2006). Angiopoietins: a link between angiogenesis and inflammation. Trends Immunol. *27*, 552-558.

Fiore,E., Fusco,C., Romero,P., and Stamenkovic,I. (2002). Matrix metalloproteinase 9 (MMP-9/gelatinase B) proteolytically cleaves ICAM-1 and participates in tumor cell resistance to natural killer cell-mediated cytotoxicity. Oncogene *21*, 5213-5223.

Fiuza,C., Bustin,M., Talwar,S., Tropea,M., Gerstenberger,E., Shelhamer,J.H., and Suffredini,A.F. (2003). Inflammation-promoting activity of HMGB1 on human microvascular endothelial cells. Blood *101*, 2652-2660.

Flaxman,A.D., Fullman,N., Otten,M.W., Jr., Menon,M., Cibulskis,R.E., Ng,M., Murray,C.J., and Lim,S.S. (2010). Rapid scaling up of insecticide-treated bed net coverage in Africa and its relationship with development assistance for health: a systematic synthesis of supply, distribution, and household survey data. PLoS. Med. *7*, e1000328.

Fowkes,F.J., Boeuf,P., and Beeson,J.G. (2016). Immunity to malaria in an era of declining malaria transmission. Parasitology *143*, 139-153.

Fowler,E.V., Peters,J.M., Gatton,M.L., Chen,N., and Cheng,Q. (2002). Genetic diversity of the DBLalpha region in *Plasmodium falciparum var* genes among Asia-Pacific isolates. Mol. Biochem. Parasitol. *120*, 117-126.

Frank,M., Dzikowski,R., Amulic,B., and Deitsch,K. (2007). Variable switching rates of malaria virulence genes are associated with chromosomal position. Mol. Microbiol. *64*, 1486-1498.

Frank,M., Dzikowski,R., Costantini,D., Amulic,B., Berdougo,E., and Deitsch,K. (2006). Strict pairing of *var* promoters and introns is required for *var* gene silencing in the malaria parasite *Plasmodium falciparum*. J. Biol. Chem. *281*, 9942-9952.

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

Freitas-Junior,L.H., Hernandez-Rivas,R., Ralph,S.A., Montiel-Condado,D., Ruvalcaba-Salazar,O.K., Rojas-Meza,A.P., Mancio-Silva,L., Leal-Silvestre,R.J., Gontijo,A.M., Shorte,S., and Scherf,A. (2005). Telomeric heterochromatin propagation and histone acetylation control mutually exclusive expression of antigenic variation genes in malaria parasites. Cell *121*, 25-36.

Fried,M. and Duffy,P.E. (1996). Adherence of *Plasmodium falciparum* to chondroitin sulfate A in the human placenta. Science *272*, 1502-1504.

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

Fry,A.E., Auburn,S., Diakite,M., Green,A., Richardson,A., Wilson,J., Jallow,M., Sisay-Joof,F., Pinder,M., Griffiths,M.J., Peshu,N., Williams,T.N., Marsh,K., Molyneux,M.E., Taylor,T.E., Rockett,K.A., and Kwiatkowski,D.P. (2008). Variation

in the ICAM1 gene is not associated with severe malaria phenotypes. Genes Immun. *9*, 462-469.

Fry,A.E., Ghansa,A., Small,K.S., Palma,A., Auburn,S., Diakite,M., Green,A., Campino,S., Teo,Y.Y., Clark,T.G., Jeffreys,A.E., Wilson,J., Jallow,M., Sisay-Joof,F., Pinder,M., Griffiths,M.J., Peshu,N., Williams,T.N., Newton,C.R., Marsh,K., Molyneux,M.E., Taylor,T.E., Koram,K.A., Oduro,A.R., Rogers,W.O., Rockett,K.A., Sabeti,P.C., and Kwiatkowski,D.P. (2009). Positive selection of a CD36 nonsense variant in sub-Saharan Africa, but no association with severe malaria phenotypes. Hum. Mol. Genet. *18*, 2683-2692.

Fuchs,S., Dohle,E., Kolbe,M., and Kirkpatrick,C.J. (2010). Outgrowth endothelial cells: sources, characteristics and potential applications in tissue engineering and regenerative medicine. Adv. Biochem. Eng Biotechnol. *123*, 201-217.

Fuchs,S., Hermanns,M.I., and Kirkpatrick,C.J. (2006). Retention of a differentiated endothelial phenotype by outgrowth endothelial cells isolated from human peripheral blood and expanded in long-term cultures. Cell Tissue Res. *326*, 79-92.

Fujinaga,H., Fujinaga,H., Watanabe,N., Kato,T., Tamano,M., Terao,M., Takada,S., Ito,Y., Umezawa,A., and Kuroda,M. (2016). Cord blood-derived endothelial colony-forming cell function is disrupted in congenital diaphragmatic hernia. Am. J. Physiol Lung Cell Mol. Physiol *310*, L1143-L1154.

Fukudome,K. and Esmon,C.T. (1994). Identification, cloning, and regulation of a novel endothelial cell protein C/activated protein C receptor. J. Biol. Chem. *269*, 26486-26491.

Fukudome,K., Kurosawa,S., Stearns-Kurosawa,D.J., He,X., Rezaie,A.R., and Esmon,C.T. (1996). The endothelial cell protein C receptor. Cell surface expression and direct ligand binding by the soluble receptor. J. Biol. Chem. *271*, 17491-17498.

Galley,H.F. and Webster,N.R. (2004). Physiology of the endothelium. Br. J. Anaesth. *93*, 105-113.

Gardner,M.J., Hall,N., Fung,E., White,O., Berriman,M., Hyman,R.W., Carlton,J.M., Pain,A., Nelson,K.E., Bowman,S., Paulsen,I.T., James,K., Eisen,J.A., Rutherford,K., Salzberg,S.L., Craig,A., Kyes,S., Chan,M.S., Nene,V., Shallom,S.J., Suh,B., Peterson,J., Angiuoli,S., Pertea,M., Allen,J., Selengut,J., Haft,D., Mather,M.W., Vaidya,A.B., Martin,D.M., Fairlamb,A.H., Fraunholz,M.J., Roos,D.S., Ralph,S.A., McFadden,G.I., Cummings,L.M., Subramanian,G.M., Mungall,C., Venter,J.C., Carucci,D.J., Hoffman,S.L., Newbold,C., Davis,R.W., Fraser,C.M., and Barrell,B. (2002). Genome sequence of the human malaria parasite *Plasmodium falciparum*. Nature *419*, 498-511.

Gazzinelli,R.T. and Denkers,E.Y. (2006). Protozoan encounters with Toll-like receptor signalling pathways: implications for host parasitism. Nat. Rev. Immunol. *6*, 895-906.

Gearing,A.J., Hemingway,I., Pigott,R., Hughes,J., Rees,A.J., and Cashman,S.J. (1992). Soluble forms of vascular adhesion molecules, E-selectin, ICAM-1, and VCAM-1: pathological significance. Ann. N. Y. Acad. Sci. *667*, 324-331.

Gearing,A.J. and Newman,W. (1993). Circulating adhesion molecules in disease. Immunol. Today *14*, 506-512.

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., and Rowe,J.A. (2012). Induction of strain-transcending antibodies against Group A PfEMP1 surface antigens from virulent malaria parasites. PLoS. Pathog. *8*, e1002665.

Ghumra,A., Semblat,J.P., McIntosh,R.S., Raza,A., Rasmussen,I.B., Braathen,R., Johansen,F.E., Sandlie,I., Mongini,P.K., Rowe,J.A., and Pleass,R.J. (2008). Identification of residues in the Cmu4 domain of polymeric IgM essential for interaction with *Plasmodium falciparum* erythrocyte membrane protein 1 (PfEMP1). J. Immunol. *181*, 1988-2000.

Gibot,S., Massin,F., Cravoisy,A., Barraud,D., Nace,L., Levy,B., and Bollaert,P.E. (2007). High-mobility group box 1 protein plasma concentrations during septic shock. Intensive Care Med. *33*, 1347-1353.

Giha,H.A., Rosthoj,S., Dodoo,D., Hviid,L., Satti,G.M., Scheike,T., Arnot,D.E., and Theander,T.G. (2000). The epidemiology of febrile malaria episodes in an area of unstable and seasonal transmission. Trans. R. Soc. Trop. Med. Hyg *94*, 645-651.

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

Glynn,J.J. and Hinds,M.T. (2014). Endothelial outgrowth cells: function and performance in vascular grafts. Tissue Eng Part B Rev. *20*, 294-303.

Goel,S., Palmkvist,M., Moll,K., Joannin,N., Lara,P., Akhouri,R.R., Moradi,N., Ojemalm,K., Westman,M., Angeletti,D., Kjellin,H., Lehtio,J., Blixt,O., Idestrom,L., Gahmberg,C.G., Storry,J.R., Hult,A.K., Olsson,M.L., von,H.G., Nilsson,I., and Wahlgren,M. (2015). RIFINs are adhesins implicated in severe *Plasmodium falciparum* malaria. Nat. Med. *21*, 314-317.

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

Grau,G.E. and Craig,A.G. (2012). Cerebral malaria pathogenesis: revisiting parasite and host contributions. Future. Microbiol. *7*, 291-302.

Grau,G.E., Taylor,T.E., Molyneux,M.E., Wirima,J.J., Vassalli,P., Hommel,M., and Lambert,P.H. (1989). Tumor necrosis factor and disease severity in children with falciparum malaria. N. Engl. J. Med. *320*, 1586-1591.

Greenwalt,D.E., Lipsky,R.H., Ockenhouse,C.F., Ikeda,H., Tandon,N.N., and Jamieson,G.A. (1992). Membrane glycoprotein CD36: a review of its roles in adherence, signal transduction, and transfusion medicine. Blood *80*, 1105-1115.

Greenwood,B., Marsh,K., and Snow,R. (1991). Why do some African children develop severe malaria? Parasitol. Today *7*, 277-281.

Griffin,J.H., Zlokovic,B.V., and Mosnier,L.O. (2012). Protein C anticoagulant and cytoprotective pathways. Int. J. Hematol. *95*, 333-345.

Griffin,J.T., Ferguson,N.M., and Ghani,A.C. (2014). Estimates of the changing age-burden of *Plasmodium falciparum* malaria disease in sub-Saharan Africa. Nat. Commun. *5*, 3136.

Griffin,J.T., Hollingsworth,T.D., Reyburn,H., Drakeley,C.J., Riley,E.M., and Ghani,A.C. (2015). Gradual acquisition of immunity to severe malaria with increasing exposure. Proc. Biol. Sci. *282*, 20142657.

Guizetti,J. and Scherf,A. (2013). Silence, activate, poise and switch! Mechanisms of antigenic variation in *Plasmodium falciparum*. Cell Microbiol. *15*, 718-726.

Gulati,R., Jevremovic,D., Peterson,T.E., Chatterjee,S., Shah,V., Vile,R.G., and Simari,R.D. (2003). Diverse origin and function of cells with endothelial phenotype obtained from adult human blood. Circ. Res. *93*, 1023-1025.

Gumina,D.L. and Su,E.J. (2017). Endothelial progenitor cells of the human placenta and fetoplacental circulation: a potential link to fetal, neonatal, and long-term health. Front Pediatr. *5*, 41.

Gupta,S. and Day,K. (1994). Clinical immunity to *Plasmodium falciparum*. Parasitol. Today *10*, 64-65.

Gupta,S., Snow,R.W., Donnelly,C.A., Marsh,K., and Newbold,C. (1999). Immunity to non-cerebral severe malaria is acquired after one or two infections. Nat. Med. *5*, 340-343.

Gyan,B., Goka,B.Q., Adjei,G.O., Tetteh,J.K., Kusi,K.A., Aikins,A., Dodoo,D., Lesser,M.L., Sison,C.P., Das,S., Howard,M.E., Milbank,E., Fischer,K., Rafii,S., Jin,D., and Golightly,L.M. (2009). Cerebral malaria is associated with low levels of circulating endothelial progenitor cells in African children. Am. J. Trop. Med. Hyg *80*, 541-546.

Hamad,A.A., El Hassan,I.M., El Khalifa,A.A., Ahmed,G.I., Abdelrahim,S.A., Theander,T.G., and Arnot,D.E. (2000). Chronic Plasmodium falciparum infections in an area of low intensity malaria transmission in the Sudan. Parasitology *120 (Pt 5)*, 447-456.

Hansen,D.S. (2012). Inflammatory responses associated with the induction of cerebral malaria: lessons from experimental murine models. PLoS. Pathog. *8*, e1003045.

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., and 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.

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

Hasler,T., Albrecht,G.R., Van Schravendijk,M.R., Aguiar,J.C., Morehead,K.E., Pasloske,B.L., Ma,C., Barnwell,J.W., Greenwood,B., and Howard,R.J. (1993). An improved microassay for *Plasmodium falciparum* cytoadherence using stable transformants of Chinese hamster ovary cells expressing CD36 or intercellular adhesion molecule-1. Am. J. Trop. Med. Hyg *48*, 332-347.

Hawkes,M., Elphinstone,R.E., Conroy,A.L., and Kain,K.C. (2013). Contrasting pediatric and adult cerebral malaria: the role of the endothelial barrier. Virulence. *4*, 543-555.

Hearn,J., Rayment,N., Landon,D.N., Katz,D.R., and de Souza,J.B. (2000). Immunopathology of cerebral malaria: morphological evidence of parasite sequestration in murine brain microvasculature. Infect. Immun. *68*, 5364-5376.

Heddini,A., Pettersson,F., Kai,O., Shafi,J., Obiero,J., Chen,Q., Barragan,A., Wahlgren,M., and Marsh,K. (2001). Fresh isolates from children with severe *Plasmodium falciparum* malaria bind to multiple receptors. Infect. Immun. *69*, 5849-5856.

Higgins,S.J., Kain,K.C., and Liles,W.C. (2011). Immunopathogenesis of falciparum malaria: implications for adjunctive therapy in the management of severe and cerebral malaria. Expert. Rev. Anti. Infect. Ther. *9*, 803-819.

Higgins,S.J., Xing,K., Kim,H., Kain,D.C., Wang,F., Dhabangi,A., Musoke,C., Cserti-Gazdewich,C.M., Tracey,K.J., Kain,K.C., and Liles,W.C. (2013). Systemic release of high mobility group box 1 (HMGB1) protein is associated with severe and fatal *Plasmodium falciparum* malaria. Malar. J. *12*, 105.

Ho,M. and White,N.J. (1999). Molecular mechanisms of cytoadherence in malaria. Am. J. Physiol *276*, C1231-C1242.

Hora,R., Kapoor,P., Thind,K.K., and Mishra,P.C. (2016). Cerebral malaria--clinical manifestations and pathogenesis. Metab Brain Dis. *31*, 225-237.

Horrocks,P., Pinches,R., Christodoulou,Z., Kyes,S.A., and Newbold,C.I. (2004). Variable *var* transition rates underlie antigenic variation in malaria. Proc. Natl. Acad. Sci. U. S. A *101*, 11129-11134.

Howard,R.J., Barnwell,J.W., Rock,E.P., Neequaye,J., Ofori-Adjei,D., Maloy,W.L., Lyon,J.A., and Saul,A. (1988). Two approximately 300 kilodalton *Plasmodium falciparum* proteins at the surface membrane of infected erythrocytes. Mol. Biochem. Parasitol. *27*, 207-223.

Howell,D.P., Levin,E.A., Springer,A.L., Kraemer,S.M., Phippard,D.J., Schief,W.R., and Smith,J.D. (2008). Mapping a common interaction site used by *Plasmodium falciparum* Duffy binding-like domains to bind diverse host receptors. Mol. Microbiol. *67*, 78-87.

Hsieh,F.L., Turner,L., Bolla,J.R., Robinson,C.V., Lavstsen,T., and Higgins,M.K. (2016). The structural basis for CD36 binding by the malaria parasite. Nat. Commun. *7*, 12837.

Hunt,N.H., Golenser,J., Chan-Ling,T., Parekh,S., Rae,C., Potter,S., Medana,I.M., Miu,J., and Ball,H.J. (2006). Immunopathogenesis of cerebral malaria. Int. J. Parasitol. *36*, 569-582.

Hunt,N.H. and Grau,G.E. (2003). Cytokines: accelerators and brakes in the pathogenesis of cerebral malaria. Trends Immunol. *24*, 491-499.

Hur,J., Yoon,C.H., Kim,H.S., Choi,J.H., Kang,H.J., Hwang,K.K., Oh,B.H., Lee,M.M., and Park,Y.B. (2004). Characterization of two types of endothelial progenitor cells and their different contributions to neovasculogenesis. Arterioscler. Thromb. Vasc. Biol. *24*, 288-293.

Hviid,L. (2005). Naturally acquired immunity to *Plasmodium falciparum* malaria in Africa. Acta Trop. *95*, 270-275.

Hviid,L. (2010). The role of Plasmodium falciparum variant surface antigens in protective immunity and vaccine development. Hum. Vaccin. *6*, 84-89.

Hviid,L. and Jensen,A.T. (2015). PfEMP1-A parasite protein family of key importance in *Plasmodium falciparum* malaria immunity and pathogenesis. Adv. Parasitol. *88*, 51-84.

Hviid,L., Theander,T.G., Elhassan,I.M., and Jensen,J.B. (1993). Increased plasma levels of soluble ICAM-1 and ELAM-1 (E-selectin) during acute *Plasmodium falciparum* malaria. Immunol. Lett. *36*, 51-58.

Idro,R., Jenkins,N.E., and Newton,C.R. (2005). Pathogenesis, clinical features, and neurological outcome of cerebral malaria. Lancet Neurol. *4*, 827-840.

Idro,R., Marsh,K., John,C.C., and Newton,C.R. (2010). Cerebral malaria: mechanisms of brain injury and strategies for improved neurocognitive outcome. Pediatr. Res. *68*, 267-274.

Ingram,D.A., Mead,L.E., Moore,D.B., Woodard,W., Fenoglio,A., and Yoder,M.C. (2005). Vessel wall-derived endothelial cells rapidly proliferate because they contain a complete hierarchy of endothelial progenitor cells. Blood *105*, 2783-2786.

Ingram,D.A., Mead,L.E., Tanaka,H., Meade,V., Fenoglio,A., Mortell,K., Pollok,K., Ferkowicz,M.J., Gilley,D., and Yoder,M.C. (2004). Identification of a novel hierarchy of endothelial progenitor cells using human peripheral and umbilical cord blood. Blood *104*, 2752-2760.

Ito,Y., Torii,Y., Ohta,R., Imai,M., Hara,S., Kawano,Y., Matsubayashi,T., Inui,A., Yoshikawa,T., Nishimura,N., Ozaki,T., Morishima,T., and Kimura,H. (2011). Increased levels of cytokines and high-mobility group box 1 are associated with the development of severe pneumonia, but not acute encephalopathy, in 2009 H1N1 influenza-infected children. Cytokine *56*, 180-187.

Jain,V., Lucchi,N.W., Wilson,N.O., Blackstock,A.J., Nagpal,A.C., Joel,P.K., Singh,M.P., Udhayakumar,V., Stiles,J.K., and Singh,N. (2011). Plasma levels of angiopoietin-1 and -2 predict cerebral malaria outcome in Central India. Malar. J. *10*, 383.

Jakobsen,P.H., Morris-Jones,S., Ronn,A., Hviid,L., Theander,T.G., Elhassan,I.M., Bygbjerg,I.C., and Greenwood,B.M. (1994). Increased plasma concentrations of sICAM-1, sVCAM-1 and sELAM-1 in patients with *Plasmodium falciparum* or *P. vivax* malaria and association with disease severity. Immunology *83*, 665-669.

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

Jenkins,N.E., Mwangi,T.W., Kortok,M., Marsh,K., Craig,A.G., and Williams,T.N. (2005). A polymorphism of intercellular adhesion molecule-1 is associated with a reduced incidence of nonmalarial febrile illness in Kenyan children. Clin. Infect. Dis. *41*, 1817-1819.

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., and Theander,T.G. (2004). *Plasmodium falciparum* associated with severe childhood malaria preferentially expresses PfEMP1 encoded by group A *var* genes. J. Exp. Med. *199*, 1179-1190.

Jensen,A.T., Zornig,H.D., Buhmann,C., Salanti,A., Koram,K.A., Riley,E.M., Theander,T.G., Hviid,L., and Staalsoe,T. (2003). Lack of gender-specific antibody recognition of products from domains of a *var* gene implicated in pregnancy-associated *Plasmodium falciparum* malaria. Infect. Immun. *71*, 4193-4196.

Jeppesen,A., Ditlev,S.B., Soroka,V., Stevenson,L., Turner,L., Dzikowski,R., Hviid,L., and Barfod,L. (2015). Multiple *Plasmodium falciparum* erythrocyte membrane protein 1 variants per genome can bind IgM via Its Fc fragment Fcmu. Infect. Immun. *83*, 3972-3981.

Jespersen,J.S., Wang,C.W., Mkumbaye,S.I., Minja,D.T., Petersen,B., Turner,L., Petersen,J.E., Lusingu,J.P., Theander,T.G., and Lavstsen,T. (2016). *Plasmodium falciparum var* genes expressed in children with severe malaria encode CIDRalpha1 domains. EMBO Mol. Med. *8*, 839-850.

Jiang,L., Mu,J., Zhang,Q., Ni,T., Srinivasan,P., Rayavara,K., Yang,W., Turner,L., Lavstsen,T., Theander,T.G., Peng,W., Wei,G., Jing,Q., Wakabayashi,Y., Bansal,A., Luo,Y., Ribeiro,J.M., Scherf,A., Aravind,L., Zhu,J., Zhao,K., and Miller,L.H. (2013). PfSETvs methylation of histone H3K36 represses virulence genes in *Plasmodium falciparum*. Nature *499*, 223-227.

Joergensen,L., Bengtsson,D.C., Bengtsson,A., Ronander,E., Berger,S.S., Turner,L., Dalgaard,M.B., Cham,G.K., Victor,M.E., Lavstsen,T., Theander,T.G., Arnot,D.E., and Jensen,A.T. (2010). Surface co-expression of two different PfEMP1 antigens on single *Plasmodium falciparum*-infected erythrocytes facilitates binding to ICAM1 and PECAM1. PLoS. Pathog. *6*, e1001083.

Joergensen,L., Vestergaard,L.S., Turner,L., Magistrado,P., Lusingu,J.P., Lemnge,M., Theander,T.G., and Jensen,A.T. (2007). 3D7-Derived *Plasmodium falciparum* erythrocyte membrane protein 1 is a frequent target of naturally acquired antibodies recognizing protein domains in a particular pattern independent of malaria transmission intensity. J. Immunol. *178*, 428-435.

Kaestli,M., Cockburn,I.A., Cortes,A., Baea,K., Rowe,J.A., and Beck,H.P. (2006). Virulence of malaria is associated with differential expression of *Plasmodium falciparum* var gene subgroups in a case-control study. J. Infect. Dis. *193*, 1567-1574.

Kaul,D.K., Liu,X.D., Nagel,R.L., and Shear,H.L. (1998). Microvascular hemodynamics and *in vivo* evidence for the role of intercellular adhesion molecule-1 in the sequestration of infected red blood cells in a mouse model of lethal malaria. Am. J. Trop. Med. Hyg *58*, 240-247.

Kendirli,T., Ince,E., Ciftci,E., Dogru,U., Egin,Y., and Akar,N. (2009). Soluble endothelial protein C receptor level in children with sepsis. Pediatr. Hematol. Oncol. *26*, 432-438.

Khattab,A., Kremsner,P.G., and Meri,S. (2013). Complement activation in primiparous women from a malaria endemic area is associated with reduced birthweight. Placenta *34*, 162-167.

Kim,H., Higgins,S., Liles,W.C., and Kain,K.C. (2011). Endothelial activation and dysregulation in malaria: a potential target for novel therapeutics. Curr. Opin. Hematol. *18*, 177-185.

Knox,T.B., Juma,E.O., Ochomo,E.O., Pates,J.H., Ndungo,L., Chege,P., Bayoh,N.M., N'Guessan,R., Christian,R.N., Hunt,R.H., and Coetzee,M. (2014). An online tool for mapping insecticide resistance in major Anopheles vectors of human malaria parasites and review of resistance status for the Afrotropical region. Parasit. Vectors. *7*, 76.

Koram,K.A., Abuaku,B., Duah,N., and Quashie,N. (2005). Comparative efficacy of antimalarial drugs including ACTs in the treatment of uncomplicated malaria among children under 5 years in Ghana. Acta Trop. *95*, 194-203.

Kraemer,S.M., Gupta,L., and Smith,J.D. (2003). New tools to identify *var* sequence tags and clone full-length genes using type-specific primers to Duffy binding-like domains. Mol. Biochem. Parasitol. *129*, 91-102.

Kraemer,S.M., Kyes,S.A., Aggarwal,G., Springer,A.L., Nelson,S.O., Christodoulou,Z., Smith,L.M., Wang,W., Levin,E., Newbold,C.I., Myler,P.J., and Smith,J.D. (2007). Patterns of gene recombination shape *var* gene repertoires in *Plasmodium falciparum*: comparisons of geographically diverse isolates. BMC. Genomics *8*, 45.

Kraemer,S.M. and Smith,J.D. (2003). Evidence for the importance of genetic structuring to the structural and functional specialization of the *Plasmodium falciparum var* gene family. Mol. Microbiol. *50*, 1527-1538.

Kraemer,S.M. and Smith,J.D. (2006). A family affair: *var* genes, PfEMP1 binding, and malaria disease. Curr. Opin. Microbiol. *9*, 374-380.

Krause,D.R., Gatton,M.L., Frankland,S., Eisen,D.P., Good,M.F., Tilley,L., and Cheng,Q. (2007). Characterization of the antibody response against *Plasmodium falciparum* erythrocyte membrane protein 1 in human volunteers. Infect. Immun. *75*, 5967-5973.

Kun,J.F., Klabunde,J., Lell,B., Luckner,D., Alpers,M., May,J., Meyer,C., and Kremsner,P.G. (1999). Association of the ICAM-1Kilifi mutation with protection against severe malaria in Lambarene, Gabon. Am. J. Trop. Med. Hyg *61*, 776-779.

Kurosawa,S., Stearns-Kurosawa,D.J., Carson,C.W., D'Angelo,A., Della,V.P., and Esmon,C.T. (1998). Plasma levels of endothelial cell protein C receptor are elevated in patients with sepsis and systemic lupus erythematosus: lack of correlation with thrombomodulin suggests involvement of different pathological processes. Blood *91*, 725-727.

Kurosawa,S., Stearns-Kurosawa,D.J., Hidari,N., and Esmon,C.T. (1997). Identification of functional endothelial protein C receptor in human plasma. J. Clin. Invest *100*, 411-418.

Kurtzhals,J.A., Adabayeri,V., Goka,B.Q., Akanmori,B.D., Oliver-Commey,J.O., Nkrumah,F.K., Behr,C., and Hviid,L. (1998). Low plasma concentrations of interleukin 10 in severe malarial anaemia compared with cerebral and uncomplicated malaria. Lancet *351*, 1768-1772.

Kweku,M., Appiah,E.K., Takramah,W., Enuameh,Y., Norman,I., and Binka,F. (2015). Effect of malaria control program on the prevalence of malaria, fever, and anaemia in children in the Hohoe municipal of Ghana: a comparative analysis of crossectional surveys. Adv. Infect. Dis. *5*, 180-188.

Kweku,M., Liu,D., Adjuik,M., Binka,F., Seidu,M., Greenwood,B., and Chandramohan,D. (2008). Seasonal intermittent preventive treatment for the prevention of anaemia and malaria in Ghanaian children: a randomized, placebo controlled trial. PLoS. One. *3*, e4000.

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

Kyes,S., Horrocks,P., and Newbold,C. (2001). Antigenic variation at the infected red cell surface in malaria. Annu. Rev. Microbiol. *55*, 673-707.

Kyes,S., Pinches,R., and Newbold,C. (2000). A simple RNA analysis method shows *var* and *rif* multigene family expression patterns in *Plasmodium falciparum*. Mol. Biochem. Parasitol. *105*, 311-315.

Kyes,S.A., Kraemer,S.M., and Smith,J.D. (2007). Antigenic variation in *Plasmodium falciparum*: gene organization and regulation of the *var* multigene family. Eukaryot. Cell *6*, 1511-1520.

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

Lamikanra,A.A., Brown,D., Potocnik,A., Casals-Pascual,C., Langhorne,J., and Roberts,D.J. (2007). Malarial anemia: of mice and men. Blood *110*, 18-28.

Langhorne,J., Ndungu,F.M., Sponaas,A.M., and Marsh,K. (2008). Immunity to malaria: more questions than answers. Nat. Immunol. *9*, 725-732.

Languino,L.R., Plescia,J., Duperray,A., Brian,A.A., Plow,E.F., Geltosky,J.E., and Altieri,D.C. (1993). Fibrinogen mediates leukocyte adhesion to vascular endothelium through an ICAM-1-dependent pathway. Cell *73*, 1423-1434.

Laszik,Z., Mitro,A., Taylor,F.B., Jr., Ferrell,G., and Esmon,C.T. (1997). Human protein C receptor is present primarily on endothelium of large blood vessels: implications for the control of the protein C pathway. Circulation *96*, 3633-3640.

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., and Higgins,M.K. (2015). Structural conservation despite huge sequence diversity allows EPCR binding by the PfEMP1 family implicated in severe childhood malaria. Cell Host. Microbe *17*, 118-129.

Lavstsen,T., Salanti,A., Jensen,A.T., Arnot,D.E., and Theander,T.G. (2003). Sub-grouping of *Plasmodium falciparum* 3D7 *var* genes based on sequence analysis of coding and non-coding regions. Malar. J. *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., and 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*, E1791-E1800.

Leech,J.H., Barnwell,J.W., Aikawa,M., Miller,L.H., and Howard,R.J. (1984a). *Plasmodium falciparum* malaria: association of knobs on the surface of infected erythrocytes with a histidine-rich protein and the erythrocyte skeleton. J. Cell Biol. *98*, 1256-1264.

Leech,J.H., Barnwell,J.W., Miller,L.H., and Howard,R.J. (1984b). Identification of a strain-specific malarial antigen exposed on the surface of *Plasmodium falciparum*-infected erythrocytes. J. Exp. Med. *159*, 1567-1575.

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., Higgins,M.K., and 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*, 403-414.

Lennartz,F., Bengtsson,A., Olsen,R.W., Joergensen,L., Brown,A., Remy,L., Man,P., Forest,E., Barfod,L.K., Adams,Y., Higgins,M.K., and Jensen,A.T. (2015). Mapping the binding site of a cross-reactive *Plasmodium falciparum* PfEMP1 monoclonal antibody inhibitory of ICAM-1 binding. J. Immunol. *195*, 3273-3283.

Liaw,P.C., Esmon,C.T., Kahnamoui,K., Schmidt,S., Kahnamoui,S., Ferrell,G., Beaudin,S., Julian,J.A., Weitz,J.I., Crowther,M., Loeb,M., and Cook,D. (2004). Patients with severe sepsis vary markedly in their ability to generate activated protein C. Blood *104*, 3958-3964.

Liles,W.C. and Kain,K.C. (2014). Endothelial activation and dysfunction in the pathogenesis of microvascular obstruction in severe malaria--a viable target for therapeutic adjunctive intervention. J. Infect. Dis. *210*, 163-164.

Lin,X., Lubinski,J.M., and Friedman,H.M. (2004). Immunization strategies to block the herpes simplex virus type 1 immunoglobulin G Fc receptor. J. Virol. *78*, 2562-2571.

Lin,Y., Chang,L., Solovey,A., Healey,J.F., Lollar,P., and Hebbel,R.P. (2002). Use of blood outgrowth endothelial cells for gene therapy for hemophilia A. Blood *99*, 457-462.

Lin,Y., Weisdorf,D.J., Solovey,A., and Hebbel,R.P. (2000). Origins of circulating endothelial cells and endothelial outgrowth from blood. J. Clin. Invest *105*, 71-77.

Lopez-Rubio,J.J., Gontijo,A.M., Nunes,M.C., Issar,N., Hernandez,R.R., and Scherf,A. (2007). 5' flanking region of *var* genes nucleate histone modification patterns linked to phenotypic inheritance of virulence traits in malaria parasites. Mol. Microbiol. *66*, 1296-1305.

Lopez-Rubio,J.J., Mancio-Silva,L., and Scherf,A. (2009). Genome-wide analysis of heterochromatin associates clonally variant gene regulation with perinuclear repressive centers in malaria parasites. Cell Host. Microbe *5*, 179-190.

Lovegrove,F.E., Tangpukdee,N., Opoka,R.O., Lafferty,E.I., Rajwans,N., Hawkes,M., Krudsood,S., Looareesuwan,S., John,C.C., Liles,W.C., and Kain,K.C. (2009). Serum angiopoietin-1 and -2 levels discriminate cerebral malaria from uncomplicated malaria and predict clinical outcome in African children. PLoS. One. *4*, e4912.

Lusingu,J.P., Jensen,A.T., Vestergaard,L.S., Minja,D.T., Dalgaard,M.B., Gesase,S., Mmbando,B.P., Kitua,A.Y., Lemnge,M.M., Cavanagh,D., Hviid,L., and Theander,T.G. (2006). Levels of plasma immunoglobulin G with specificity against the cysteine-rich interdomain regions of a semiconserved *Plasmodium falciparum* erythrocyte membrane protein 1, VAR4, predict protection against malarial anemia and febrile episodes. Infect. Immun. *74*, 2867-2875.

Lusingu,J.P., Vestergaard,L.S., Mmbando,B.P., Drakeley,C.J., Jones,C., Akida,J., Savaeli,Z.X., Kitua,A.Y., Lemnge,M.M., and Theander,T.G. (2004). Malaria morbidity and immunity among residents of villages with different *Plasmodium falciparum* transmission intensity in North-Eastern Tanzania. Malar. J. *3*, 26.

Lyke,K.E., Burges,R., Cissoko,Y., Sangare,L., Dao,M., Diarra,I., Kone,A., Harley,R., Plowe,C.V., Doumbo,O.K., and Sztein,M.B. (2004). Serum levels of the proinflammatory cytokines interleukin-1 beta (IL-1beta), IL-6, IL-8, IL-10, tumor necrosis factor alpha, and IL-12(p70) in Malian children with severe *Plasmodium falciparum* malaria and matched uncomplicated malaria or healthy controls. Infect. Immun. *72*, 5630-5637.

Mackintosh,C.L., Beeson,J.G., and Marsh,K. (2004). Clinical features and pathogenesis of severe malaria. Trends Parasitol. *20*, 597-603.

Mackintosh,C.L., Christodoulou,Z., Mwangi,T.W., Kortok,M., Pinches,R., Williams,T.N., Marsh,K., and Newbold,C.I. (2008). Acquisition of naturally occurring antibody responses to recombinant protein domains of *Plasmodium falciparum* erythrocyte membrane protein 1. Malar. J. *7*, 155.

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

Magistrado,P.A., Lusingu,J., Vestergaard,L.S., Lemnge,M., Lavstsen,T., Turner,L., Hviid,L., Jensen,A.T., and Theander,T.G. (2007). Immunoglobulin G antibody reactivity to a group A *Plasmodium falciparum* erythrocyte membrane protein 1 and protection from *P. falciparum* malaria. Infect. Immun. *75*, 2415-2420.

Magistrado,P.A., Minja,D., Doritchamou,J., Ndam,N.T., John,D., Schmiegelow,C., Massougbodji,A., Dahlback,M., Ditlev,S.B., Pinto,V.V., Resende,M., Lusingu,J., Theander,T.G., Salanti,A., and Nielsen,M.A. (2011). High efficacy of anti DBL4varepsilon-VAR2CSA antibodies in inhibition of CSA-binding *Plasmodium falciparum*-infected erythrocytes from pregnant women. Vaccine *29*, 437-443.

Marlin,S.D. and Springer,T.A. (1987). Purified intercellular adhesion molecule-1 (ICAM-1) is a ligand for lymphocyte function-associated antigen 1 (LFA-1). Cell *51*, 813-819.

Marsh,K., English,M., Crawley,J., and Peshu,N. (1996). The pathogenesis of severe malaria in African children. Ann. Trop. Med. Parasitol. *90*, 395-402.

Marsh,K., Forster,D., Waruiru,C., Mwangi,I., Winstanley,M., Marsh,V., Newton,C., Winstanley,P., Warn,P., Peshu,N., and . (1995). Indicators of life-threatening malaria in African children. N. Engl. J. Med. *332*, 1399-1404.

Marti,M., Good,R.T., Rug,M., Knuepfer,E., and Cowman,A.F. (2004). Targeting malaria virulence and remodeling proteins to the host erythrocyte. Science *306*, 1930-1933.

Martin-Ramirez,J., Hofman,M., van den Biggelaar,M., Hebbel,R.P., and Voorberg,J. (2012). Establishment of outgrowth endothelial cells from peripheral blood. Nat. Protoc. *7*, 1709-1715.

Martin-Ramirez,J., Kok,M.G., Hofman,M., Bierings,R., Creemers,E.E., Meijers,J.C., Voorberg,J., and Pinto-Sietsma,S.J. (2014). Individual with subclinical atherosclerosis have impaired proliferation of blood outgrowth endothelial cells, which can be restored by statin therapy. PLoS. One. *9*, e99890.

Matsushita,K., Yamakuchi,M., Morrell,C.N., Ozaki,M., O'Rourke,B., Irani,K., and Lowenstein,C.J. (2005). Vascular endothelial growth factor regulation of Weibel-Palade-body exocytosis. Blood *105*, 207-214.

Mayor,A., Rovira-Vallbona,E., Srivastava,A., Sharma,S.K., Pati,S.S., Puyol,L., Quinto,L., Bassat,Q., Machevo,S., Mandomando,I., Chauhan,V.S., Alonso,P.L., and Chitnis,C.E. (2009). Functional and immunological characterization of a Duffy binding-like alpha domain from *Plasmodium falciparum* erythrocyte membrane protein 1 that mediates rosetting. Infect. Immun. *77*, 3857-3863.

McCormick,C.J., Craig,A., Roberts,D., Newbold,C.I., and Berendt,A.R. (1997). Intercellular adhesion molecule-1 and CD36 synergize to mediate adherence of *Plasmodium falciparum*-infected erythrocytes to cultured human microvascular endothelial cells. J. Clin. Invest *100*, 2521-2529.

McGilvray,I.D., Serghides,L., Kapus,A., Rotstein,O.D., and Kain,K.C. (2000). Nonopsonic monocyte/macrophage phagocytosis of *Plasmodium falciparum*-parasitized erythrocytes: a role for CD36 in malarial clearance. Blood *96*, 3231-3240.

McGregor,I.A., Williams,K., and Goodwin,L.G. (1963). Pyrimethamine and Sulphadiazine in treatment of malaria. Br. Med. J. *2*, 728-729.

McGuire,W., Hill,A.V., Greenwood,B.M., and Kwiatkowski,D. (1996). Circulating ICAM-1 levels in *falciparum* malaria are high but unrelated to disease severity. Trans. R. Soc. Trop. Med. Hyg *90*, 274-276.

Medana,I.M., Chaudhri,G., Chan-Ling,T., and Hunt,N.H. (2001). Central nervous system in cerebral malaria: 'Innocent bystander' or active participant in the induction of immunopathology? Immunol. Cell Biol. *79*, 101-120.

Medana,I.M. and Turner,G.D. (2006). Human cerebral malaria and the blood-brain barrier. Int. J. Parasitol. *36*, 555-568.

Medina,E., Molinari,G., Rohde,M., Haase,B., Chhatwal,G.S., and Guzman,C.A. (1999). Fc-mediated nonspecific binding between fibronectin-binding protein I of *Streptococcus pyogenes* and human immunoglobulins. J. Immunol. *163*, 3396-3402.

Medina,P., Navarro,S., Estelles,A., Vaya,A., Bertina,R.M., and Espana,F. (2005). Influence of the 4600A/G and 4678G/C polymorphisms in the endothelial protein C receptor (EPCR) gene on the risk of venous thromboembolism in carriers of factor V Leiden. Thromb. Haemost. *94*, 389-394.

Medina,P., Navarro,S., Estelles,A., Vaya,A., Woodhams,B., Mira,Y., Villa,P., Migaud-Fressart,M., Ferrando,F., Aznar,J., Bertina,R.M., and Espana,F. (2004). Contribution of polymorphisms in the endothelial protein C receptor gene to soluble endothelial protein C receptor and circulating activated protein C levels, and thrombotic risk. Thromb. Haemost. *91*, 905-911.

Medina,R.J., O'Neill,C.L., Humphreys,M.W., Gardiner,T.A., and Stitt,A.W. (2010). Outgrowth endothelial cells: characterization and their potential for reversing ischemic retinopathy. Invest Ophthalmol. Vis. Sci. *51*, 5906-5913.

Medina,R.J., O'Neill,C.L., O'Doherty,T.M., Chambers,S.E., Guduric-Fuchs,J., Neisen,J., Waugh,D.J., Simpson,D.A., and Stitt,A.W. (2013). *Ex vivo* expansion of human outgrowth endothelial cells leads to IL-8-mediated replicative senescence and impaired vasoreparative function. Stem Cells *31*, 1657-1668.

Melcher,M., Muhle,R.A., Henrich,P.P., Kraemer,S.M., Avril,M., Vigan-Womas,I., Mercereau-Puijalon,O., Smith,J.D., and Fidock,D.A. (2010). Identification of a role for the PfEMP1 semi-conserved head structure in protein trafficking to the surface of *Plasmodium falciparum* infected red blood cells. Cell Microbiol. *12*, 1446-1462.

Merrick,C.J., Huttenhower,C., Buckee,C., Amambua-Ngwa,A., Gomez-Escobar,N., Walther,M., Conway,D.J., and Duraisingh,M.T. (2012). Epigenetic dysregulation of virulence gene expression in severe *Plasmodium falciparum* malaria. J. Infect. Dis. *205*, 1593-1600.

Miller,L.H., Ackerman,H.C., Su,X.Z., and Wellems,T.E. (2013). Malaria biology and disease pathogenesis: insights for new treatments. Nat. Med. *19*, 156-167.

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

Miller,L.H., Good,M.F., and Milon,G. (1994). Malaria pathogenesis. Science *264*, 1878-1883.

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., and Taylor,T.E. (2015). Quantitative assessment of multiorgan

sequestration of parasites in fatal pediatric cerebral malaria. J. Infect. Dis. *212*, 1317-1321.

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

Mkumbaye,S.I., Wang,C.W., Lyimo,E., Jespersen,J.S., Manjurano,A., Mosha,J., Kavishe,R.A., Mwakalinga,S.B., Minja,D.T., Lusingu,J.P., Theander,T.G., and Lavstsen,T. (2017). The severity of *Plasmodium falciparum* infection is associated with transcript levels of *var* genes encoding endothelial protein C receptor-binding *P. falciparum* erythrocyte membrane protein 1. Infect. Immun. *85*.

MOH (2014a). Ministry of Health, Ghana: National Malaria Control Programme 2013. Annual Report. 2013.

MOH (2014b). MOH . Ministry of Health: Strategic plan for malaria control in Ghana. 2014 - 2020.

Molyneux,M.E. (1989). Malaria--clinical features in children. J. R. Soc. Med. *82 Suppl 17*, 35-38.

Mota,M.M. and Rodriguez,A. (2008). New pieces for the malaria liver stage puzzle: where will they fit? Cell Host. Microbe *3*, 63-65.

Moussiliou,A., Alao,M.J., Denoeud-Ndam,L., Tahar,R., Ezimegnon,S., Sagbo,G., Amoussou,A., Luty,A.J., Deloron,P., and Tuikue,N.N. (2015). High plasma levels of soluble endothelial protein C receptor are associated with increased mortality among children with cerebral malaria in Benin. J. Infect. Dis. *211*, 1484-1488.

Moxon,C.A., Chisala,N.V., Wassmer,S.C., Taylor,T.E., Seydel,K.B., Molyneux,M.E., Faragher,B., Kennedy,N., Toh,C.H., Craig,A.G., and Heyderman,R.S. (2014). Persistent endothelial activation and inflammation after *Plasmodium falciparum* Infection in Malawian children. J. Infect. Dis. *209*, 610-615.

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., and Heyderman,R.S. (2013). Loss of endothelial protein C receptors links coagulation and inflammation to parasite sequestration in cerebral malaria in African children. Blood *122*, 842-851.

Muanza,K., Gay,F., Behr,C., and Scherf,A. (1996). Primary culture of human lung microvessel endothelial cells: a useful *in vitro* model for studying *Plasmodium falciparum*-infected erythrocyte cytoadherence. Res. Immunol. *147*, 149-163.

Muellenbeck,M.F., Ueberheide,B., Amulic,B., Epp,A., Fenyo,D., Busse,C.E., Esen,M., Theisen,M., Mordmuller,B., and Wardemann,H. (2013). Atypical and

classical memory B cells produce Plasmodium falciparum neutralizing antibodies. J. Exp. Med. *210*, 389-399.

Mutunga,M., Fulton,B., Bullock,R., Batchelor,A., Gascoigne,A., Gillespie,J.I., and Baudouin,S.V. (2001). Circulating endothelial cells in patients with septic shock. Am. J. Respir. Crit Care Med. *163*, 195-200.

Naing,C., Whittaker,M.A., Nyunt,W., V, and Mak,J.W. (2014). Is *Plasmodium vivax* malaria a severe malaria?: a systematic review and meta-analysis. PLoS. Negl. Trop. Dis. *8*, e3071.

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

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

Niang,M., Bei,A.K., Madnani,K.G., Pelly,S., Dankwa,S., Kanjee,U., Gunalan,K., Amaladoss,A., Yeo,K.P., Bob,N.S., Malleret,B., Duraisingh,M.T., and Preiser,P.R. (2014). STEVOR is a Plasmodium falciparum erythrocyte binding protein that mediates merozoite invasion and rosetting. Cell Host. Microbe *16*, 81-93.

Nielsen,M.A., Pinto,V.V., Resende,M., Dahlback,M., Ditlev,S.B., Theander,T.G., and Salanti,A. (2009). Induction of adhesion-inhibitory antibodies against placental Plasmodium falciparum parasites by using single domains of VAR2CSA. Infect. Immun. *77*, 2482-2487.

Nielsen,M.A., Staalsoe,T., Kurtzhals,J.A., Goka,B.Q., Dodoo,D., Alifrangis,M., Theander,T.G., Akanmori,B.D., and Hviid,L. (2002). *Plasmodium falciparum* variant surface antigen expression varies between isolates causing severe and nonsevere malaria and is modified by acquired immunity. J. Immunol. *168*, 3444-3450.

Nkumama,I.N., O'Meara,W.P., and Osier,F.H. (2017). Changes in malaria epidemiology in Africa and new challenges for elimination. Trends Parasitol. *33*, 128-140.

Noedl,H., Se,Y., Schaecher,K., Smith,B.L., Socheat,D., and Fukuda,M.M. (2008). Evidence of artemisinin-resistant malaria in western Cambodia. N. Engl. J. Med. *359*, 2619-2620.

Nonvignon,J., Aryeetey,G.C., Malm,K.L., Agyemang,S.A., Aubyn,V.N., Peprah,N.Y., Bart-Plange,C.N., and Aikins,M. (2016). Economic burden of malaria on businesses in Ghana: a case for private sector investment in malaria control. Malar. J. *15*, 454.

Nunes-Silva,S., Dechavanne,S., Moussiliou,A., Pstrag,N., Semblat,J.P., Gangnard,S., Tuikue-Ndam,N., Deloron,P., Chene,A., and Gamain,B. (2015). Beninese children

with cerebral malaria do not develop humoral immunity against the IT4-VAR19-DC8 PfEMP1 variant linked to EPCR and brain endothelial binding. Malar. J. *14*, 493.

Obonyo,C.O., Vulule,J., Akhwale,W.S., and Grobbee,D.E. (2007). In-hospital morbidity and mortality due to severe malarial anemia in western Kenya. Am. J. Trop. Med. Hyg *77*, 23-28.

Ochola,L.B., Siddondo,B.R., Ocholla,H., Nkya,S., Kimani,E.N., Williams,T.N., Makale,J.O., Liljander,A., Urban,B.C., Bull,P.C., Szestak,T., Marsh,K., and Craig,A.G. (2011). Specific receptor usage in *Plasmodium falciparum* cytoadherence is associated with disease outcome. PLoS. One. *6*, e14741.

Ockenhouse,C.F., Betageri,R., Springer,T.A., and 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*, 63-69.

Ockenhouse,C.F., Ho,M., Tandon,N.N., Van Seventer,G.A., Shaw,S., White,N.J., Jamieson,G.A., Chulay,J.D., and Webster,H.K. (1991). Molecular basis of sequestration in severe and uncomplicated *Plasmodium falciparum* malaria: differential adhesion of infected erythrocytes to CD36 and ICAM-1. J. Infect. Dis. *164*, 163-169.

Oduro (2015). The role of circulating endothelial progenitor cells (CEPCs) and other biomarkers in the pathogenesis of cerebral malaria . PhD Dissertation *University of Ghana, Accra, Ghana*.

Ofori,M.F., Dodoo,D., Staalsoe,T., Kurtzhals,J.A., Koram,K., Theander,T.G., Akanmori,B.D., and Hviid,L. (2002). Malaria-induced acquisition of antibodies to *Plasmodium falciparum* variant surface antigens. Infect. Immun. *70*, 2982-2988.

Oleinikov,A.V., Amos,E., Frye,I.T., Rossnagle,E., Mutabingwa,T.K., Fried,M., and Duffy,P.E. (2009). High throughput functional assays of the variant antigen PfEMP1 reveal a single domain in the 3D7 *Plasmodium falciparum* genome that binds ICAM1 with high affinity and is targeted by naturally acquired neutralizing antibodies. PLoS. Pathog. *5*, e1000386.

Omi,K., Ohashi,J., Patarapotikul,J., Hananantachai,H., Naka,I., Looareesuwan,S., and Tokunaga,K. (2003). CD36 polymorphism is associated with protection from cerebral malaria. Am. J. Hum. Genet. *72*, 364-374.

Oquendo,P., Hundt,E., Lawler,J., and Seed,B. (1989). CD36 directly mediates cytoadherence of *Plasmodium falciparum* parasitized erythrocytes. Cell *58*, 95-101.

Orem,J.N., Kirigia,J.M., Azairwe,R., Kasirye,I., and Walker,O. (2012). Impact of malaria morbidity on gross domestic product in Uganda. Int. Arch. Med. *5*, 12.

Ormiston,M.L., Toshner,M.R., Kiskin,F.N., Huang,C.J., Groves,E., Morrell,N.W., and Rana,A.A. (2015). Generation and culture of blood outgrowth endothelial cells from human peripheral blood. J. Vis. Exp. e53384.

Pain,A., Ferguson,D.J., Kai,O., Urban,B.C., Lowe,B., Marsh,K., and 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*, 1805-1810.

Park,G.S., Ireland,K.F., Opoka,R.O., and John,C.C. (2012). Evidence of endothelial activation in asymptomatic *Plasmodium falciparum* parasitemia and effect of blood group on levels of von Willebrand factor in malaria. J. Pediatric. Infect. Dis. Soc. *1*, 16-25.

Park,J.S., Gamboni-Robertson,F., He,Q., Svetkauskaite,D., Kim,J.Y., Strassheim,D., Sohn,J.W., Yamada,S., Maruyama,I., Banerjee,A., Ishizaka,A., and Abraham,E. (2006). High mobility group box 1 protein interacts with multiple Toll-like receptors. Am. J. Physiol Cell Physiol *290*, C917-C924.

Pate,M., Damarla,V., Chi,D.S., Negi,S., and Krishnaswamy,G. (2010). Endothelial cell biology: role in the inflammatory response. Adv. Clin. Chem. *52*, 109-130.

Perez-Toledo,K., Rojas-Meza,A.P., Mancio-Silva,L., Hernandez-Cuevas,N.A., Delgadillo,D.M., Vargas,M., Martinez-Calvillo,S., Scherf,A., and Hernandez-Rivas,R. (2009). *Plasmodium falciparum* heterochromatin protein 1 binds to tri-methylated histone 3 lysine 9 and is linked to mutually exclusive expression of *var* genes. Nucleic Acids Res. *37*, 2596-2606.

Peters,J., Fowler,E., Gatton,M., Chen,N., Saul,A., and Cheng,Q. (2002). High diversity and rapid changeover of expressed *var* genes during the acute phase of *Plasmodium falciparum* infections in human volunteers. Proc. Natl. Acad. Sci. U. S. A *99*, 10689-10694.

Petersen,J.E., Bouwens,E.A., Tamayo,I., Turner,L., Wang,C.W., Stins,M., Theander,T.G., Hermida,J., Mosnier,L.O., and Lavstsen,T. (2015). Protein C system defects inflicted by the malaria parasite protein PfEMP1 can be overcome by a soluble EPCR variant. Thromb. Haemost. *114*, 1038-1048.

Petter,M. and Duffy,M.F. (2015). Antigenic Variation in *Plasmodium falciparum*. Results Probl. Cell Differ. *57*, 47-90.

Petter,M., Lee,C.C., Byrne,T.J., Boysen,K.E., Volz,J., Ralph,S.A., Cowman,A.F., Brown,G.V., and Duffy,M.F. (2011). Expression of *P. falciparum var* genes involves exchange of the histone variant H2A.Z at the promoter. PLoS. Pathog. *7*, e1001292.

Pleass,R.J., Moore,S.C., Stevenson,L., and Hviid,L. (2016). Immunoglobulin M: restrainer of inflammation and mediator of immune evasion by *Plasmodium falciparum* alaria. Trends Parasitol. *32*, 108-119.

Pongponratn,E., Riganti,M., Punpoowong,B., and Aikawa,M. (1991). Microvascular sequestration of parasitized erythrocytes in human falciparum malaria: a pathological study. Am. J. Trop. Med. Hyg *44*, 168-175.

Pongponratn,E., Turner,G.D., Day,N.P., Phu,N.H., Simpson,J.A., Stepniewska,K., Mai,N.T., Viriyavejakul,P., Looareesuwan,S., Hien,T.T., Ferguson,D.J., and White,N.J. (2003). An ultrastructural study of the brain in fatal *Plasmodium falciparum* malaria. Am. J. Trop. Med. Hyg *69*, 345-359.

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., and Turner,G.D. (2012). Sequestration and microvascular congestion are associated with coma in human cerebral malaria. J. Infect. Dis. *205*, 663-671.

Portugal,S., Pierce,S.K., and Crompton,P.D. (2013). Young lives lost as B cells falter: what we are learning about antibody responses in malaria. J. Immunol. *190*, 3039-3046.

Prapansilp,P., Medana,I., Mai,N.T., Day,N.P., Phu,N.H., Yeo,T.W., Hien,T.T., White,N.J., Anstey,N.M., and Turner,G.D. (2013). A clinicopathological correlation of the expression of the angiopoietin-Tie-2 receptor pathway in the brain of adults with *Plasmodium falciparum* malaria. Malar. J. *12*, 50.

Przyborski,J.M., Nyboer,B., and Lanzer,M. (2016). Ticket to ride: export of proteins to the *Plasmodium falciparum*-infected erythrocyte. Mol. Microbiol. *101*, 1-11.

Qu,D., Wang,Y., Esmon,N.L., and Esmon,C.T. (2007). Regulated endothelial protein C receptor shedding is mediated by tumor necrosis factor-alpha converting enzyme/ADAM17. J. Thromb. Haemost. *5*, 395-402.

Quashie,N.B., Duah,N.O., Abuaku,B., Quaye,L., Ayanful-Torgby,R., Akwoviah,G.A., Kweku,M., Johnson,J.D., Lucchi,N.W., Udhayakumar,V., Duplessis,C., Kronmann,K.C., and Koram,K.A. (2013). A SYBR Green 1-based in vitro test of susceptibility of Ghanaian *Plasmodium falciparum* clinical isolates to a panel of anti-malarial drugs. Malar. J. *12*, 450.

Ralph,S.A., Scheidig-Benatar,C., and Scherf,A. (2005). Antigenic variation in *Plasmodium falciparum* is associated with movement of *var* loci between subnuclear locations. Proc. Natl. Acad. Sci. U. S. A *102*, 5414-5419.

Ralph,S.A. and Scherf,A. (2005). The epigenetic control of antigenic variation in *Plasmodium falciparum*. Curr. Opin. Microbiol. *8*, 434-440.

Rao,L.V., Esmon,C.T., and Pendurthi,U.R. (2014). Endothelial cell protein C receptor: a multiliganded and multifunctional receptor. Blood *124*, 1553-1562.

Rask,T.S., Hansen,D.A., Theander,T.G., Gorm,P.A., and Lavstsen,T. (2010). *Plasmodium falciparum* erythrocyte membrane protein 1 diversity in seven genomes--divide and conquer. PLoS. Comput. Biol. *6*.

Rasti,N., Namusoke,F., Chene,A., Chen,Q., Staalsoe,T., Klinkert,M.Q., Mirembe,F., Kironde,F., and Wahlgren,M. (2006). Nonimmune immunoglobulin binding and multiple adhesion characterize *Plasmodium falciparum*-infected erythrocytes of placental origin. Proc. Natl. Acad. Sci. U. S. A *103*, 13795-13800.

Recker,M., Buckee,C.O., Serazin,A., Kyes,S., Pinches,R., Christodoulou,Z., Springer,A.L., Gupta,S., and Newbold,C.I. (2011). Antigenic variation in *Plasmodium falciparum* malaria involves a highly structured switching pattern. PLoS. Pathog. *7*, e1001306.

Regan,L.M., Stearns-Kurosawa,D.J., Kurosawa,S., Mollica,J., Fukudome,K., and Esmon,C.T. (1996). The endothelial cell protein C receptor. Inhibition of activated protein C anticoagulant function without modulation of reaction with proteinase inhibitors. J. Biol. Chem. *271*, 17499-17503.

Reyburn,H., Mbatia,R., Drakeley,C., Bruce,J., Carneiro,I., Olomi,R., Cox,J., Nkya,W.M., Lemnge,M., Greenwood,B.M., and Riley,E.M. (2005). Association of transmission intensity and age with clinical manifestations and case fatality of severe *Plasmodium falciparum* malaria. JAMA *293*, 1461-1470.

Roberts,D.J., Craig,A.G., Berendt,A.R., Pinches,R., Nash,G., Marsh,K., and Newbold,C.I. (1992). Rapid switching to multiple antigenic and adhesive phenotypes in malaria. Nature *357*, 689-692.

Roberts,D.J., Pain,A., Kai,O., Kortok,M., and Marsh,K. (2000). Autoagglutination of malaria-infected red blood cells and malaria severity. Lancet *355*, 1427-1428.

Robinson,B.A., Welch,T.L., and Smith,J.D. (2003). Widespread functional specialization of *Plasmodium falciparum* erythrocyte membrane protein 1 family members to bind CD36 analysed across a parasite genome. Mol. Microbiol. *47*, 1265-1278.

Rogerson,S.J., Chaiyaroj,S.C., Ng,K., Reeder,J.C., and Brown,G.V. (1995). Chondroitin sulfate A is a cell surface receptor for *Plasmodium falciparum*-infected erythrocytes. J. Exp. Med. *182*, 15-20.

Rogerson,S.J., Tembenu,R., Dobano,C., Plitt,S., Taylor,T.E., and Molyneux,M.E. (1999). Cytoadherence characteristics of *Plasmodium falciparum*-infected erythrocytes from Malawian children with severe and uncomplicated malaria. Am. J. Trop. Med. Hyg *61*, 467-472.

Rogier,C., Tall,A., Diagne,N., Fontenille,D., Spiegel,A., and Trape,J.F. (1999). *Plasmodium falciparum* clinical malaria: lessons from longitudinal studies in Senegal. Parassitologia *41*, 255-259.

Romer,L.H., McLean,N.V., Yan,H.C., Daise,M., Sun,J., and DeLisser,H.M. (1995). IFN-gamma and TNF-alpha induce redistribution of PECAM-1 (CD31) on human endothelial cells. J. Immunol. *154*, 6582-6592.

Rosenberg,E., Ben-Shmuel,A., Shalev,O., Sinay,R., Cowman,A., and Pollack,Y. (2009). Differential, positional-dependent transcriptional response of antigenic variation (*var*) genes to biological stress in *Plasmodium falciparum*. PLoS. One. *4*, e6991.

Rottmann,M., Lavstsen,T., Mugasa,J.P., Kaestli,M., Jensen,A.T., Muller,D., Theander,T., and Beck,H.P. (2006). Differential expression of *var* gene groups is associated with morbidity caused by *Plasmodium falciparum* infection in Tanzanian children. Infect. Immun. *74*, 3904-3911.

Rovira-Vallbona,E., Moncunill,G., Bassat,Q., Aguilar,R., Machevo,S., Puyol,L., Quinto,L., Menendez,C., Chitnis,C.E., Alonso,P.L., Dobano,C., and Mayor,A. (2012). Low antibodies against *Plasmodium falciparum* and imbalanced pro-inflammatory cytokines are associated with severe malaria in Mozambican children: a case-control study. Malar. J. *11*, 181.

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

Rowe,J.A., Shafi,J., Kai,O.K., Marsh,K., and Raza,A. (2002). Nonimmune IgM, but not IgG binds to the surface of *Plasmodium falciparum*-infected erythrocytes and correlates with rosetting and severe malaria. Am. J. Trop. Med. Hyg *66*, 692-699.

RTS,S. (2015). Efficacy and safety of RTS,S/AS01 malaria vaccine with or without a booster dose in infants and children in Africa: final results of a phase 3, individually randomised, controlled trial. Lancet *386*, 31-45.

Russell,T.L., Govella,N.J., Azizi,S., Drakeley,C.J., Kachur,S.P., and Killeen,G.F. (2011). Increased proportions of outdoor feeding among residual malaria vector populations following increased use of insecticide-treated nets in rural Tanzania. Malar. J. *10*, 80.

Sabchareon,A., Burnouf,T., Quattara,D., Attanath,P., Bouharoun-Tayoun,H., Chantavanich,P., Foucault,C., Chongsuphajaisiddhi,T., and Druilhe,P. (1991). Parasitologic and clinical human response to immunoglobulin administration in *falciparum* malaria. Am. J. Trop. Med. Hyg *45*, 297-308.

Sachs,J. and Malaney,P. (2002). The economic and social burden of malaria. Nature *415*, 680-685.

Sahu,P.K., Satpathi,S., Behera,P.K., Mishra,S.K., Mohanty,S., and Wassmer,S.C. (2015). Pathogenesis of cerebral malaria: new diagnostic tools, biomarkers, and therapeutic approaches. Front Cell Infect. Microbiol. *5*, 75.

Sakamoto,T.M., Lanaro,C., Ozelo,M.C., Garrido,V.T., Olalla-Saad,S.T., Conran,N., and Costa,F.F. (2013). Increased adhesive and inflammatory properties in blood outgrowth endothelial cells from sickle cell anemia patients. Microvasc. Res. *90*, 173-179.

Salanti,A., Dahlback,M., Turner,L., Nielsen,M.A., Barfod,L., Magistrado,P., Jensen,A.T., Lavstsen,T., Ofori,M.F., Marsh,K., Hviid,L., and Theander,T.G. (2004). Evidence for the involvement of VAR2CSA in pregnancy-associated malaria. J. Exp. Med. *200*, 1197-1203.

Salanti,A., Staalsoe,T., Lavstsen,T., Jensen,A.T., Sowa,M.P., Arnot,D.E., Hviid,L., and Theander,T.G. (2003). Selective upregulation of a single distinctly structured *var* gene in chondroitin sulphate A-adhering *Plasmodium falciparum* involved in pregnancy-associated malaria. Mol. Microbiol. *49*, 179-191.

Saposnik,B., Peynaud-Debayle,E., Stepanian,A., Baron,G., Simansour,M., Mandelbrot,L., de,P.D., and Gandrille,S. (2012). Elevated soluble endothelial cell protein C receptor (sEPCR) levels in women with preeclampsia: a marker of endothelial activation/damage? Thromb. Res. *129*, 152-157.

Scaffidi,P., Misteli,T., and Bianchi,M.E. (2002). Release of chromatin protein HMGB1 by necrotic cells triggers inflammation. Nature *418*, 191-195.

Scherf,A., Hernandez-Rivas,R., Buffet,P., Bottius,E., Benatar,C., Pouvelle,B., Gysin,J., and Lanzer,M. (1998). Antigenic variation in malaria: *in situ* switching, relaxed and mutually exclusive transcription of var genes during intra-erythrocytic development in *Plasmodium falciparum*. EMBO J. *17*, 5418-5426.

Scherf,A., Lopez-Rubio,J.J., and Riviere,L. (2008). Antigenic variation in *Plasmodium falciparum*. Annu. Rev. Microbiol. *62*, 445-470.

Schindelin,J., Arganda-Carreras,I., Frise,E., Kaynig,V., Longair,M., Pietzsch,T., Preibisch,S., Rueden,C., Saalfeld,S., Schmid,B., Tinevez,J.Y., White,D.J., Hartenstein,V., Eliceiri,K., Tomancak,P., and Cardona,A. (2012). Fiji: an open-source platform for biological-image analysis. Nat. Methods *9*, 676-682.

Schmidt,J.A., Udeinya,I.J., Leech,J.H., Hay,R.J., Aikawa,M., Barnwell,J., Green,I., and 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*, 379-386.

Schofield,L. and Grau,G.E. (2005). Immunological processes in malaria pathogenesis. Nat. Rev. Immunol. *5*, 722-735.

Schofield,L. and Mueller,I. (2006). Clinical immunity to malaria. Curr. Mol. Med. *6*, 205-221.

Scholander,C., Carlson,J., Kremsner,P.G., and Wahlgren,M. (1998). Extensive immunoglobulin binding of *Plasmodium falciparum*-infected erythrocytes in a group of children with moderate anemia. Infect. Immun. *66*, 361-363.

Scholander,C., Treutiger,C.J., Hultenby,K., and Wahlgren,M. (1996). Novel fibrillar structure confers adhesive property to malaria-infected erythrocytes. Nat. Med. *2*, 204-208.

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

Semblat,J.P., Raza,A., Kyes,S.A., and Rowe,J.A. (2006). Identification of *Plasmodium falciparum* var1CSA and var2CSA domains that bind IgM natural antibodies. Mol. Biochem. Parasitol. *146*, 192-197.

Serghides,L., Smith,T.G., Patel,S.N., and Kain,K.C. (2003). CD36 and malaria: friends or foes? Trends Parasitol. *19*, 461-469.

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., and Taylor,T.E. (2015). Brain swelling and death in children with cerebral malaria. N. Engl. J. Med. *372*, 1126-1137.

Seydel,K.B., Milner,D.A., Jr., Kamiza,S.B., Molyneux,M.E., and Taylor,T.E. (2006). The distribution and intensity of parasite sequestration in comatose Malawian children. J. Infect. Dis. *194*, 208-5.

Shabani,E., Opoka,R.O., Bangirana,P., Park,G.S., Vercellotti,G.M., Guan,W., Hodges,J.S., Lavstsen,T., and John,C.C. (2016). The endothelial protein C receptor rs867186-GG genotype is associated with increased soluble EPCR and could mediate protection against severe malaria. Sci. Rep. *6*, 27084.

Shikani,H.J., Freeman,B.D., Lisanti,M.P., Weiss,L.M., Tanowitz,H.B., and Desruisseaux,M.S. (2012). Cerebral malaria: we have come a long way. Am. J. Pathol. *181*, 1484-1492.

Siciliano,G. and Alano,P. (2015). Enlightening the malaria parasite life cycle: bioluminescent *Plasmodium* in fundamental and applied research. Front Microbiol. *6*, 391.

Silamut,K., Phu,N.H., Whitty,C., Turner,G.D., Louwrier,K., Mai,N.T., Simpson,J.A., Hien,T.T., and White,N.J. (1999). A quantitative analysis of the microvascular sequestration of malaria parasites in the human brain. Am. J. Pathol. *155*, 395-410.

Silvie,O., Mota,M.M., Matuschewski,K., and Prudencio,M. (2008). Interactions of the malaria parasite and its mammalian host. Curr. Opin. Microbiol. *11*, 352-359.

Simmons,D., Makgoba,M.W., and Seed,B. (1988). ICAM, an adhesion ligand of LFA-1, is homologous to the neural cell adhesion molecule NCAM. Nature *331*, 624-627.

Sinka,M.E., Bangs,M.J., Manguin,S., Coetzee,M., Mbogo,C.M., Hemingway,J., Patil,A.P., Temperley,W.H., Gething,P.W., Kabaria,C.W., Okara,R.M., Van,B.T., Godfray,H.C., Harbach,R.E., and Hay,S.I. (2010). The dominant Anopheles vectors of human malaria in Africa, Europe and the Middle East: occurrence data, distribution maps and bionomic precis. Parasit. Vectors. *3*, 117.

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

Smith,J.D., Craig,A.G., Kriek,N., Hudson-Taylor,D., Kyes,S., Fagan,T., Pinches,R., Baruch,D.I., Newbold,C.I., and Miller,L.H. (2000). Identification of a *Plasmodium falciparum* intercellular adhesion molecule-1 binding domain: a parasite adhesion trait implicated in cerebral malaria. Proc. Natl. Acad. Sci. U. S. A *97*, 1766-1771.

Snounou,G., Zhu,X., Siripoon,N., Jarra,W., Thaithong,S., Brown,K.N., and Viriyakosol,S. (1999). Biased distribution of msp1 and msp2 allelic variants in *Plasmodium falciparum* populations in Thailand. Trans. R. Soc. Trop. Med. Hyg *93*, 369-374.

Snow,R.W., Bastos,d.A., I, Lowe,B.S., Kabiru,E.W., Nevill,C.G., Mwankusye,S., Kassiga,G., Marsh,K., and Teuscher,T. (1994). Severe childhood malaria in two areas of markedly different falciparum transmission in east Africa. Acta Trop. *57*, 289-300.

Snow,R.W., Guerra,C.A., Noor,A.M., Myint,H.Y., and Hay,S.I. (2005). The global distribution of clinical episodes of Plasmodium falciparum malaria. Nature *434*, 214-217.

Soerli,J., Barfod,L., Lavstsen,T., Bernasconi,N.L., Lanzavecchia,A., and Hviid,L. (2009). Human monoclonal IgG selection of *Plasmodium falciparum* for the expression of placental malaria-specific variant surface antigens. Parasite Immunol. *31*, 341-346.

Solovey,A., Lin,Y., Browne,P., Choong,S., Wayner,E., and Hebbel,R.P. (1997). Circulating activated endothelial cells in sickle cell anemia. N. Engl. J. Med. *337*, 1584-1590.

Staunton,D.E., Merluzzi,V.J., Rothlein,R., Barton,R., Marlin,S.D., and Springer,T.A. (1989). A cell adhesion molecule, ICAM-1, is the major surface receptor for rhinoviruses. Cell *56*, 849-853.

Stearns-Kurosawa,D.J., Kurosawa,S., Mollica,J.S., Ferrell,G.L., and Esmon,C.T. (1996). The endothelial cell protein C receptor augments protein C activation by the thrombin-thrombomodulin complex. Proc. Natl. Acad. Sci. U. S. A *93*, 10212-10216.

Steketee,R.W., Nahlen,B.L., Parise,M.E., and Menendez,C. (2001). The burden of malaria in pregnancy in malaria-endemic areas. Am. J. Trop. Med. Hyg *64*, 28-35.

Stevenson,L., Huda,P., Jeppesen,A., Laursen,E., Rowe,J.A., Craig,A., Streicher,W., Barfod,L., and Hviid,L. (2015a). Investigating the function of Fc-specific binding of IgM to *Plasmodium falciparum* erythrocyte membrane protein 1 mediating erythrocyte rosetting. Cell Microbiol. *17*, 819-831.

Stevenson,L., Laursen,E., Cowan,G.J., Bandoh,B., Barfod,L., Cavanagh,D.R., Andersen,G.R., and Hviid,L. (2015b). alpha2-Macroglobulin can crosslink multiple *Plasmodium falciparum* Erythrocyte Membrane Protein 1 (PfEMP1) molecules and may facilitate adhesion of parasitized erythrocytes. PLoS. Pathog. *11*, e1005022.

Stevenson,M.M. and Riley,E.M. (2004). Innate immunity to malaria. Nat. Rev. Immunol. *4*, 169-180.

Storm,J. and Craig,A.G. (2014). Pathogenesis of cerebral malaria--inflammation and cytoadherence. Front Cell Infect. Microbiol. *4*, 100.

Strijbos,M.H., Landburg,P.P., Nur,E., Teerlink,T., Leebeek,F.W., Rijneveld,A.W., Biemond,B.J., Sleijfer,S., Gratama,J.W., Duits,A.J., and Schnog,J.J. (2009). Circulating endothelial cells: a potential parameter of organ damage in sickle cell anemia? Blood Cells Mol. Dis. *43*, 63-67.

Su,X.Z., Heatwole,V.M., Wertheimer,S.P., Guinet,F., Herrfeldt,J.A., Peterson,D.S., Ravetch,J.A., and 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*, 89-100.

Suguitan,A.L., Jr., Cadigan,T.J., Nguyen,T.A., Zhou,A., Leke,R.J., Metenou,S., Thuita,L., Megnekou,R., Fogako,J., Leke,R.G., and Taylor,D.W. (2003). Malaria-associated cytokine changes in the placenta of women with pre-term deliveries in Yaounde, Cameroon. Am. J. Trop. Med. Hyg *69*, 574-581.

Sunden-Cullberg,J., Norrby-Teglund,A., Rouhiainen,A., Rauvala,H., Herman,G., Tracey,K.J., Lee,M.L., Andersson,J., Tokics,L., and Treutiger,C.J. (2005). Persistent

elevation of high mobility group box-1 protein (HMGB1) in patients with severe sepsis and septic shock. Crit Care Med. *33*, 564-573.

Swamy,L., Amulic,B., and Deitsch,K.W. (2011). *Plasmodium falciparum var* gene silencing is determined by cis DNA elements that form stable and heritable interactions. Eukaryot. Cell *10*, 530-539.

Swerlick,R.A., Lee,K.H., Wick,T.M., and Lawley,T.J. (1992). Human dermal microvascular endothelial but not human umbilical vein endothelial cells express CD36 *in vivo* and *in vitro*. J. Immunol. *148*, 78-83.

Szoke,K., Reinisch,A., Ostrup,E., Reinholt,F.P., and Brinchmann,J.E. (2016). Autologous cell sources in therapeutic vasculogenesis: *In vitro* and *in vivo* comparison of endothelial colony-forming cells from peripheral blood and endothelial cells isolated from adipose tissue. Cytotherapy. *18*, 242-252.

Takala,S.L., Coulibaly,D., Thera,M.A., Batchelor,A.H., Cummings,M.P., Escalante,A.A., Ouattara,A., Traore,K., Niangaly,A., Djimde,A.A., Doumbo,O.K., and Plowe,C.V. (2009). Extreme polymorphism in a vaccine antigen and risk of clinical malaria: implications for vaccine development. Sci. Transl. Med. *1*, 2ra5.

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

Tchinda,V.H., Tadem,A.D., Tako,E.A., Tene,G., Fogako,J., Nyonglema,P., Sama,G., Zhou,A., and Leke,R.G. (2007). Severe malaria in Cameroonian children: correlation between plasma levels of three soluble inducible adhesion molecules and TNF-αalpha. Acta Trop. *102*, 20-28.

Teasdale,G. and Jennett,B. (1974). Assessment of coma and impaired consciousness. A practical scale. Lancet *2*, 81-84.

Tebo,A.E., Kremsner,P.G., Piper,K.P., and Luty,A.J. (2002). Low antibody responses to variant surface antigens of *Plasmodium falciparum* are associated with severe malaria and increased susceptibility to malaria attacks in Gabonese children. Am. J. Trop. Med. Hyg *67*, 597-603.

Theisen,M., Soe,S., Jessing,S.G., Okkels,L.M., Danielsen,S., Oeuvray,C., Druilhe,P., and Jepsen,S. (2000). Identification of a major B-cell epitope of the *Plasmodium falciparum* glutamate-rich protein (GLURP), targeted by human antibodies mediating parasite killing. Vaccine *19*, 204-212.

Timmermans,F., Van,H.F., De,S.M., Raedt,R., Plasschaert,F., De Buyzere,M.L., Gillebert,T.C., Plum,J., and Vandekerckhove,B. (2007). Endothelial outgrowth cells

are not derived from CD133+ cells or CD45+ hematopoietic precursors. Arterioscler. Thromb. Vasc. Biol. *27*, 1572-1579.

Tonkin,C.J., Carret,C.K., Duraisingh,M.T., Voss,T.S., Ralph,S.A., Hommel,M., Duffy,M.F., Silva,L.M., Scherf,A., Ivens,A., Speed,T.P., Beeson,J.G., and Cowman,A.F. (2009). Sir2 paralogues cooperate to regulate virulence genes and antigenic variation in *Plasmodium falciparum*. PLoS. Biol. *7*, e84.

Trimnell,A.R., Kraemer,S.M., Mukherjee,S., Phippard,D.J., Janes,J.H., Flamoe,E., Su,X.Z., Awadalla,P., and Smith,J.D. (2006). Global genetic diversity and evolution of *var* genes associated with placental and severe childhood malaria. Mol. Biochem. Parasitol. *148*, 169-180.

Tse,M.T., Chakrabarti,K., Gray,C., Chitnis,C.E., and Craig,A. (2004). Divergent binding sites on intercellular adhesion molecule-1 (ICAM-1) for variant *Plasmodium falciparum* isolates. Mol. Microbiol. *51*, 1039-1049.

Tun,K.M., Imwong,M., Lwin,K.M., Win,A.A., Hlaing,T.M., Hlaing,T., Lin,K., Kyaw,M.P., Plewes,K., Faiz,M.A., Dhorda,M., Cheah,P.Y., Pukrittayakamee,S., Ashley,E.A., Anderson,T.J., Nair,S., McDew-White,M., Flegg,J.A., Grist,E.P., Guerin,P., Maude,R.J., Smithuis,F., Dondorp,A.M., Day,N.P., Nosten,F., White,N.J., and Woodrow,C.J. (2015). Spread of artemisinin-resistant Plasmodium falciparum in Myanmar: a cross-sectional survey of the K13 molecular marker. Lancet Infect. Dis. *15*, 415-421.

Tura,O., Skinner,E.M., Barclay,G.R., Samuel,K., Gallagher,R.C., Brittan,M., Hadoke,P.W., Newby,D.E., Turner,M.L., and Mills,N.L. (2013). Late outgrowth endothelial cells resemble mature endothelial cells and are not derived from bone marrow. Stem Cells *31*, 338-348.

Turner,G.D., Ly,V.C., Nguyen,T.H., Tran,T.H., Nguyen,H.P., Bethell,D., Wyllie,S., Louwrier,K., Fox,S.B., Gatter,K.C., Day,N.P., Tran,T.H., White,N.J., and Berendt,A.R. (1998). Systemic endothelial activation occurs in both mild and severe malaria. Correlating dermal microvascular endothelial cell phenotype and soluble cell adhesion molecules with disease severity. Am. J. Pathol. *152*, 1477-1487.

Turner,G.D., Morrison,H., Jones,M., Davis,T.M., Looareesuwan,S., Buley,I.D., Gatter,K.C., Newbold,C.I., Pukritayakamee,S., Nagachinta,B., and . (1994). An immunohistochemical study of the pathology of fatal malaria. Evidence for widespread endothelial activation and a potential role for intercellular adhesion molecule-1 in cerebral sequestration. Am. J. Pathol. *145*, 1057-1069.

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., and Theander,T.G. (2013). Severe malaria is associated with parasite binding to endothelial protein C receptor. Nature *498*, 502-505.

Turner,L., Lavstsen,T., Mmbando,B.P., Wang,C.W., Magistrado,P.A., Vestergaard,L.S., Ishengoma,D.S., Minja,D.T., Lusingu,J.P., and Theander,T.G. (2015). IgG antibodies to endothelial protein C receptor-binding cysteine-rich interdomain region domains of *Plasmodium falciparum* erythrocyte membrane protein 1 are acquired early in life in individuals exposed to malaria. Infect. Immun. *83*, 3096-3103.

Tuteja,R. (2007). Malaria - an overview. FEBS J. *274*, 4670-4679.

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

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

Udomsangpetch,R., Chivapat,S., Viriyavejakul,P., Riganti,M., Wilairatana,P., Pongponratin,E., and Looareesuwan,S. (1997). Involvement of cytokines in the histopathology of cerebral malaria. Am. J. Trop. Med. Hyg *57*, 501-506.

Uitte de,W.S., Van,M., V, Rosendaal,F.R., Vos,H.L., de Visser,M.C., and Bertina,R.M. (2004). Haplotypes of the EPCR gene, plasma sEPCR levels and the risk of deep venous thrombosis. J. Thromb. Haemost. *2*, 1305-1310.

Ukaegbu,U.E., Kishore,S.P., Kwiatkowski,D.L., Pandarinath,C., Dahan-Pasternak,N., Dzikowski,R., and Deitsch,K.W. (2014). Recruitment of PfSET2 by RNA polymerase II to variant antigen encoding loci contributes to antigenic variation in *P. falciparum*. PLoS. Pathog. *10*, e1003854.

Ukaegbu,U.E., Zhang,X., Heinberg,A.R., Wele,M., Chen,Q., and Deitsch,K.W. (2015). A unique virulence gene occupies a principal position in immune evasion by the malaria parasite *Plasmodium falciparum*. PLoS. Genet. *11*, e1005234.

Urban,B.C., Ferguson,D.J., Pain,A., Willcox,N., Plebanski,M., Austyn,J.M., and Roberts,D.J. (1999). *Plasmodium falciparum*-infected erythrocytes modulate the maturation of dendritic cells. Nature *400*, 73-77.

Urban,B.C., Ing,R., and Stevenson,M.M. (2005). Early interactions between blood-stage *Plasmodium* parasites and the immune system. Curr. Top. Microbiol. Immunol. *297*, 25-70.

van Beem,R.T., Verloop,R.E., Kleijer,M., Noort,W.A., Loof,N., Koolwijk,P., van der Schoot,C.E., van Hinsbergh,V.W., and Zwaginga,J.J. (2009). Blood outgrowth endothelial cells from cord blood and peripheral blood: angiogenesis-related characteristics *in vitro*. J. Thromb. Haemost. *7*, 217-226.

van de Stolpe,A. and van der Saag,P.T. (1996). Intercellular adhesion molecule-1. J. Mol. Med. (Berl) *74*, 13-33.

van der Heyde,H.C., Nolan,J., Combes,V., Gramaglia,I., and Grau,G.E. (2006). A unified hypothesis for the genesis of cerebral malaria: sequestration, inflammation and hemostasis leading to microcirculatory dysfunction. Trends Parasitol. *22*, 503-508.

van Hensbroek,M.B., Palmer,A., Onyiorah,E., Schneider,G., Jaffar,S., Dolan,G., Memming,H., Frenkel,J., Enwere,G., Bennett,S., Kwiatkowski,D., and Greenwood,B. (1996). The effect of a monoclonal antibody to tumor necrosis factor on survival from childhood cerebral malaria. J. Infect. Dis. *174*, 1091-1097.

Volz,J.C., Bartfai,R., Petter,M., Langer,C., Josling,G.A., Tsuboi,T., Schwach,F., Baum,J., Rayner,J.C., Stunnenberg,H.G., Duffy,M.F., and Cowman,A.F. (2012). PfSET10, a *Plasmodium falciparum* methyltransferase, maintains the active *var* gene in a poised state during parasite division. Cell Host. Microbe *11*, 7-18.

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

Walliker,D., Quakyi,I.A., Wellems,T.E., McCutchan,T.F., Szarfman,A., London,W.T., Corcoran,L.M., Burkot,T.R., and Carter,R. (1987). Genetic analysis of the human malaria parasite *Plasmodium falciparum*. Science *236*, 1661-1666.

Wang,C.W., Lavstsen,T., Bengtsson,D.C., Magistrado,P.A., Berger,S.S., Marquard,A.M., Alifrangis,M., Lusingu,J.P., Theander,T.G., and Turner,L. (2012). Evidence for *in vitro* and *in vivo* expression of the conserved VAR3 (type 3) *Plasmodium falciparum* erythrocyte membrane protein 1. Malar. J. *11*, 129.

Wang,H., Bloom,O., Zhang,M., Vishnubhakat,J.M., Ombrellino,M., Che,J., Frazier,A., Yang,H., Ivanova,S., Borovikova,L., Manogue,K.R., Faist,E., Abraham,E., Andersson,J., Andersson,U., Molina,P.E., Abumrad,N.N., Sama,A., and Tracey,K.J. (1999a). HMG-1 as a late mediator of endotoxin lethality in mice. Science *285*, 248-251.

Wang,H., Vishnubhakat,J.M., Bloom,O., Zhang,M., Ombrellino,M., Sama,A., and Tracey,K.J. (1999b). Proinflammatory cytokines (tumor necrosis factor and interleukin 1) stimulate release of high mobility group protein-1 by pituicytes. Surgery *126*, 389-392.

Wang,H., Yang,H., Czura,C.J., Sama,A.E., and Tracey,K.J. (2001). HMGB1 as a late mediator of lethal systemic inflammation. Am. J. Respir. Crit Care Med. *164*, 1768-1773.

Wang,H.E., Shapiro,N.I., Griffin,R., Safford,M.M., Judd,S., and Howard,G. (2013a). Inflammatory and endothelial activation biomarkers and risk of sepsis: a nested case-control study. J. Crit Care *28*, 549-555.

Wang,J.W., Bouwens,E.A., Pintao,M.C., Voorberg,J., Safdar,H., Valentijn,K.M., de Boer,H.C., Mertens,K., Reitsma,P.H., and Eikenboom,J. (2013b). Analysis of the storage and secretion of von Willebrand factor in blood outgrowth endothelial cells derived from patients with von Willebrand disease. Blood *121*, 2762-2772.

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

Warimwe,G.M., Keane,T.M., Fegan,G., Musyoki,J.N., Newton,C.R., Pain,A., Berriman,M., Marsh,K., and Bull,P.C. (2009). *Plasmodium falciparum var* gene expression is modified by host immunity. Proc. Natl. Acad. Sci. U. S. A *106*, 21801-21806.

Wassmer,S.C., Cianciolo,G.J., Combes,V., and Grau,G.E. (2005). Inhibition of endothelial activation: a new way to treat cerebral malaria? PLoS. Med. *2*, e245.

Wassmer,S.C., Combes,V., Candal,F.J., Juhan-Vague,I., and Grau,G.E. (2006). Platelets potentiate brain endothelial alterations induced by *Plasmodium falciparum*. Infect. Immun. *74*, 645-653.

Wassmer,S.C. and Grau,G.E. (2017). Severe malaria: what's new on the pathogenesis front? Int. J. Parasitol. *47*, 145-152.

Wassmer,S.C., Moxon,C.A., Taylor,T., Grau,G.E., Molyneux,M.E., and Craig,A.G. (2011). Vascular endothelial cells cultured from patients with cerebral or uncomplicated malaria exhibit differential reactivity to TNF. Cell Microbiol. *13*, 198-209.

Weiss,G.E., Clark,E.H., Li,S., Traore,B., Kayentao,K., Ongoiba,A., Hernandez,J.N., Doumbo,O.K., Pierce,S.K., Branch,O.H., and Crompton,P.D. (2011). A positive correlation between atypical memory B cells and *Plasmodium falciparum* transmission intensity in cross-sectional studies in Peru and Mali. PLoS. One. *6*, e15983.

White,N.J., Pukrittayakamee,S., Hien,T.T., Faiz,M.A., Mokuolu,O.A., and Dondorp,A.M. (2014). Malaria. Lancet *383*, 723-735.

White,N.J., Turner,G.D., Medana,I.M., Dondorp,A.M., and Day,N.P. (2010). The murine cerebral malaria phenomenon. Trends Parasitol. *26*, 11-15.

White,V.A. (2011). Malaria in Malawi: inside a research autopsy study of pediatric cerebral malaria. Arch. Pathol. Lab Med. *135*, 220-226.

WHO (2000). Severe falciparum malaria. In Transactions of the Royal Society of Tropical Medicine and Hygiene 1-90.

WHO (2016). World malaria report 2016. http://www. who. int/malaria/publications/world-malaria-report-2016/report/en/.

Xu,J., Qu,D., Esmon,N.L., and Esmon,C.T. (2000). Metalloproteolytic release of endothelial cell protein C receptor. J. Biol. Chem. *275*, 6038-6044.

Yeo,T.W., Lampah,D.A., Gitawati,R., Tjitra,E., Kenangalem,E., Piera,K., Price,R.N., Duffull,S.B., Celermajer,D.S., and Anstey,N.M. (2008). Angiopoietin-2 is associated with decreased endothelial nitric oxide and poor clinical outcome in severe *falciparum* malaria. Proc. Natl. Acad. Sci. U. S. A *105*, 17097-17102.

Yeo,T.W., Lampah,D.A., Kenangalem,E., Tjitra,E., Weinberg,J.B., Granger,D.L., Price,R.N., and Anstey,N.M. (2014). Decreased endothelial nitric oxide bioavailability, impaired microvascular function, and increased tissue oxygen consumption in children with falciparum malaria. J. Infect. Dis. *210*, 1627-1632.

Yeo,T.W., Lampah,D.A., Tjitra,E., Piera,K., Gitawati,R., Kenangalem,E., Price,R.N., and Anstey,N.M. (2010). Greater endothelial activation, Weibel-Palade body release and host inflammatory response to *Plasmodium vivax*, compared with *Plasmodium falciparum*: a prospective study in Papua, Indonesia. J. Infect. Dis. *202*, 109-112.

Yipp,B.G., Anand,S., Schollaardt,T., Patel,K.D., Looareesuwan,S., and Ho,M. (2000). Synergism of multiple adhesion molecules in mediating cytoadherence of *Plasmodium* falciparum-infected erythrocytes to microvascular endothelial cells under flow. Blood *96*, 2292-2298.

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

Yoder,M.C., Mead,L.E., Prater,D., Krier,T.R., Mroueh,K.N., Li,F., Krasich,R., Temm,C.J., Prchal,J.T., and Ingram,D.A. (2007). Redefining endothelial progenitor cells via clonal analysis and hematopoietic stem/progenitor cell principals. Blood *109*, 1801-1809.

Yoon,Y.S., Uchida,S., Masuo,O., Cejna,M., Park,J.S., Gwon,H.C., Kirchmair,R., Bahlman,F., Walter,D., Curry,C., Hanley,A., Isner,J.M., and Losordo,D.W. (2005). Progressive attenuation of myocardial vascular endothelial growth factor expression is a seminal event in diabetic cardiomyopathy: restoration of microvascular homeostasis and recovery of cardiac function in diabetic cardiomyopathy after replenishment of local vascular endothelial growth factor. Circulation *111*, 2073-2085.

Zhang,Q., Huang,Y., Zhang,Y., Fang,X., Claes,A., Duchateau,M., Namane,A., Lopez-Rubio,J.J., Pan,W., and Scherf,A. (2011a). A critical role of perinuclear filamentous

actin in spatial repositioning and mutually exclusive expression of virulence genes in malaria parasites. Cell Host. Microbe *10*, 451-463.

Zhang,Q., Siegel,T.N., Martins,R.M., Wang,F., Cao,J., Gao,Q., Cheng,X., Jiang,L., Hon,C.C., Scheidig-Benatar,C., Sakamoto,H., Turner,L., Jensen,A.T., Claes,A., Guizetti,J., Malmquist,N.A., and Scherf,A. (2014). Exonuclease-mediated degradation of nascent RNA silences genes linked to severe malaria. Nature *513*, 431-435.

Zhang,Q., Zhang,Y., Huang,Y., Xue,X., Yan,H., Sun,X., Wang,J., McCutchan,T.F., and Pan,W. (2011b). From *in vivo* to *in vitro*: dynamic analysis of *Plasmodium falciparum var* gene expression patterns of patient isolates during adaptation to culture. PLoS. One. *6*, e20591.

Zhang,Y., Ingram,D.A., Murphy,M.P., Saadatzadeh,M.R., Mead,L.E., Prater,D.N., and Rehman,J. (2009). Release of proinflammatory mediators and expression of proinflammatory adhesion molecules by endothelial progenitor cells. Am. J. Physiol Heart Circ. Physiol *296*, H1675-H1682.

APPENDICES

Appendix A

Ethical Clearance

GHANA HEALTH SERVICE ETHICAL REVIEW COMMITTEE

In case of reply the number and date of this Letter should be quoted.

My Ref. :GHS-ERC: 3
Your Ref. No.

Research & Development Division
Ghana Health Service
P. O. Box MB 190
Accra
Tel: +233-302-681109
Fax + 233-302-685424
Email: Hannah. Frimpong@ghsmail.org

24th June, 2014

Dr. Michael Fokuo Ofori
NMIMR
University of Ghana
Accra

ETHICAL APPROVAL - ID NO: GHS-ERC: 08/05/14

The Ghana Health Service Ethics Review Committee has reviewed and given approval for the implementation of your Study Protocol titled:

"Malaria vaccine research and capacity building (Mavareca): Profile of clinical malaria cases in the Hohoe district"

This approval requires that you inform the Ethical Review Committee (ERC) when the study begins and provide Mid-term reports of the study to the Ethical Review Committee (ERC) for continuous review. The ERC may observe or cause to be observed procedures and records of the study during and after implementation.

Please note that any modification without ERC approval is rendered invalid.

You are also required to report all serious adverse events related to this study to the ERC within seven days verbally and fourteen days in writing.

You are requested to submit a final report on the study to assure the ERC that the project was implemented as per approved protocol. You are also to inform the ERC and your sponsor before any publication of the research findings.

Please always quote the protocol identification number in all future correspondence in relation to this approved protocol

SIGNED.....
DR. CYNTHIA BANNERMAN
(GHS-ERC VICE-CHAIRPERSON)

Cc: The Director, Research & Development Division, Ghana Health Service, Accra

Appendix B

Primers for amplification of PfEMP1-specific DNA sequences and production of recombinant proteins

Recombinant protein in *E. coli*			
	Gene ID	Genome	Primers (F: Forward; R: Reverse)
DBL_D4 (b3)	PFD1235w/PF3D7_0425800	3D7	Published in Bengtsson *et al.* 2013b
DBL_D5 (b3)	PFD1235w/PF3D7_0425800	3D7	Published in Bengtsson *et al.* 2013b
Motif	PFD1235w/PF3D7_0425800	3D7	F: 5´-GCGGATCCTTATACGCAAAAGCACG-3´
			R: 5´-CTAgctagc*tta*GGGTGTTGTATCATCAG-3´
D5_motif	PFD1235w/PF3D7_0425800	3D7	PCR1
			F: 5´-GAGGATTTATACGCAAAAGCACGA-3´
			R: 5´-TGCAGCAGTAGAATACACACTATACACAGTGGGTGTTGT-3´
			PCR2
			F: 5´-GAAGATCT AATCCGTGTGCTG -3´
			R: 5´-TCGTGCTTTTGCGTATAAATCCTC-3´
			PCR3
			F: 5´-ACAACACCCACTGTGTATAGTGTGTATTCTACTGCTGCA-3´
			R: 5´-CTAgctagc*tta*GCAATCACACGC-3´
			PCR4
			F: 5´-GAAGATCTAATCCGTGTGCTG-3´

			R: 5´-TGCAGCAGTAGAATACACACTATACACAGTGGGTGTTGT-3´
			PCR5
			F: 5´-GAGGATTTATACGCAAAAGCACGA-3´
			R: 5´-CTAgctagc*tta*GCAATCACACGC-3´
			PCR6
			F: 5´-GAAGATCTAATCCGTGTGCTG -3´
			R: 5´-CTAgctagc*tta*GCAATCACACGC-3´
DBL_D4 (b3)	PF11_0521/ PF3D7_1150400	3D7	F: 5´-GAAGATCTAACCCGTGTGCTAAACCTC-3´
			R: 5´-CTAGCTAGC*tta*GCATTTACATGCTGTATC-3´
DBL_D4 (b3)	JF712900	BM021	Published in Bengtsson *et al.* 2013b
DBL_D4 (b3)	JF712901	BM028	Published in Bengtsson *et al.* 2013b
DBL_D4 (b3)	JF712902	BM048	Published in Bengtsson *et al.* 2013b
DBL_D4 (b3)	JF712903	BM066	Published in Bengtsson *et al.* 2013b
DBL_D4 (b3)	JN037695	BM057	Published in Bengtsson *et al.* 2013b
DBL_D4 (b1)	Dd2var32 KOB85388	Dd2	Published in Bengtsson *et al.* 2013b
DBL_D4 (b11)	DD2var25	Dd2	F: 5´-CTAgctagcAACCCGTGTGTTAAACCTCG-3´
			R: 5´-CTAGCTAGC*tta*ACAACCACACGCACCATC-3´

DBL_D4 (b7)	DD2var52	Dd2	F: 5´-GCGGATCCAATCCCTGCGTTACTGGTG-3´
			R: 5´-CTAGCTAGC*tta*ACAAGTACACTGATCTTTATA-3´
DBL_D4 (b3)	HB3var03 KOB63865	HB3	F: 5´-GCGGATCCAACTCGTGTGGAAAGAACAC-3´
			R: 5´-CTAgctagc*tta*GCAACGTAACGCCTCATC-3´
DBL_D4 (b7)	HB3var01	HB3	F: 5´-GCGGATCCAATCCGTGTGTCAATGGTG-3´
			R: 5´-GCGGATCC*tta*ACAAATACACGCCTCATC-3´
DBL_D4 (b11)	HB3var1csa	HB3	5´-CTAgctagcAACCCGTGTGTTAAACCTCG-3´
			5´-CTAGCTAGC*tta*ACAACCACACGCACCATC-3´
DBL_D4 (b1)	AFJ66668	BT1914	F: 5´-GAAGATCTAACCCGTGTGGAAAGAACAC-3´
			R: 5´-GAAGATCT*tta*ACATGCACACGGCGTTTCAT-3´
DBL_D4 (bx)	KC608962	pap1	Synthetic gene produced by Eurofins
DBL_D4 (b1)	KF984156	MN062	F: 5´-GCGGATCCAACCCGTGTGTAAAAAACAATAATG-3´
			R: 5´-CTAGCTAGC*tta*GCAACGTAACGCCTCATC-3´
DBL_D4 (b3)	KJ866957	MN035	F: 5´-GCGGATCCAACCCCTGTGCAAAGAACACTAC-3´
			R: CTAGCTAGC*tta*ACATTCGCAAGCTTTTTC-

			3´
DBL_D4 (b3)	KJ866958	-	F: 5´-GCGGATCCAATCCTTGTGGACATAAAAGTG-3´
			R: 5´-CTAGCTAGC*tta*ACAACCTAACGCATAATC-3´
DBL_D4 (b1)	KM364031	MN056	F: 5´-GCGGATCCAACCCCTGTGCAAAGAACAC-3´
			R: 5´-CTAGCTAGC*tta*ACAACCACACGCCTCATC-3´
DBL_D4 (b3)	KM364033	-	F: 5´-GCGGATCCAATCCTTGTGGACATAAAAGTG-3´
			R: 5´-CTAGCTAGC*tta*ACAACCTAACGCATAATC-3´
DBL_D4 (b12)	JQ691646	BT1983	F: 5´-CTAgctagcAACCCCTGTGGAAAAAAGAAT-3´
			R: 5´-CTAgctagc*tta*ACACTTACACGCCTCATC-3´
DBL_D4 (b3)	JQ691647	BT1983	F: 5´-GCGGATCCAATCCATGCGTTAATGGTC-3´
			R: 5´-CTAgctagc*tta*ACACGTACATTTCGCATG-3´
DBL_D4 (b6)	JQ691649	BT1983	F: 5´-GCGGATCCAATCCCTGCGTTAATGGTC-3´
			R: 5´-GCGGATCC*tta*ACATTTACATGCCACATC-3´
DBL_D4 (b3)	KJ866959	-	F: 5´-GCGGATCCAATCCTTGTGGACATAAAAGTG-3´
			R: 5´-

			ctaGCTAGCttaACAACCTAACGCATAATC-3´
DBL_D4 (b6)	KM364034	MN035	F: 5´-GCGGATCCAATCCGTGTGTAAACACCACTG-3´
			R: 5´-CTAGCTAGC*tta*ACACGTACATTTCGCATG-3´
DBL_D4 (b5)	IT4var13	IT4	F: 5´-GCGGATCCAATCCCTGTGTAGTTGGA-3´
			R: 5´-CTAGCTAGC*tta*ACATTTACATGCATTAGC-3´
CIDR_D3 (a1.6)-DBL_D4 (b3)	PFD1235w/PF3D7_0425800	3D7	F: 5´-GCGGATCCCCTGAATGTGGAGTCCAATG-3´
			R: 5´CTAgctagc*tta*ACACTTACACGCCTCAAC
Recombinant protein in insect cells			
CIDR_D6 (b4)	PF11_0008/PF3D7_1100200	3D7	F: 5´-GAATTCAAAAAACAAGAAAAACTATAT-3´
			R: 5´-TGCGGCCGCTACATGGATTTGCTGGAACA-3´
DBL_D2 (a1.7)-CIDR_D3 (a1.4)-DBL_D4 (b3)	PF11_0521/ PF3D7_1150400	3D7	F: 5´-CTCTAGAAATGGGGAATGCAATACCAGCGACTCCGGAT-3´
			R: 5´-CTGCGGCCGCTGCATTTACATGCTGTATCATGATCATGTGG-3´
DBL_D2 (a1.7)-CIDR_D3 (a1.4)-DBL_D4 (b1)	Dd2var32 KOB85388	Dd2	F: 5´-CTCGAATTCatgggggaaattcttcaaaaggtgctcct-3´
			R: 5´-CTGCGGCCGCTccacctgattatgaaaaagcttgcgaatgt-3´

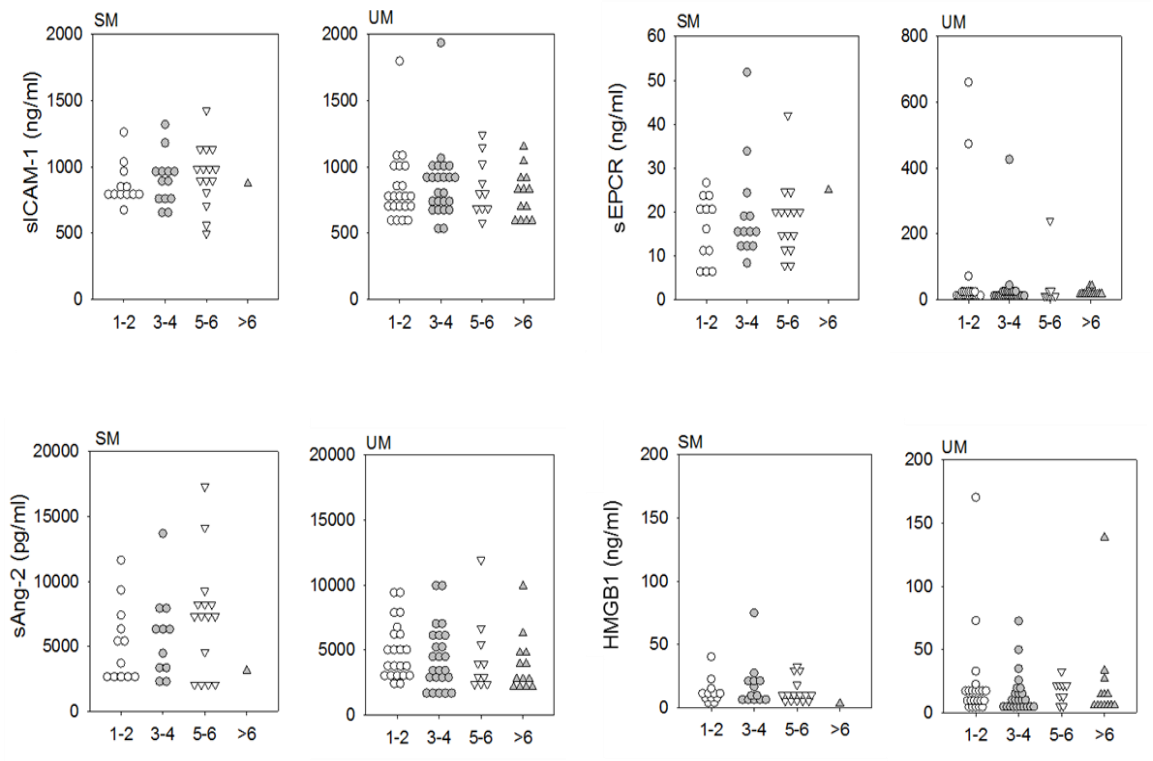
Full length	IT4var13	IT4	Published in Turner *et al*. 2013
Other primers used			
M3637	-	-	F: 5´-TgctNKtaatggtggtcc-3´
M3638	-	-	R: 5´-TGCCACCATTTTGTA-3´
DBLα2/1.1/2/4/7)	–	-	F: 5'-GATTAYGTBCCTCAATTTTTAMGWTGGT
			R: 5' TACAATCATATCCATTAWGACTACAA
			R: 5'-TCACAATCGCATCCATTATGACTACAA
Q0183	-	-	F: 5´-AATTNHTGCTNKTAATGGTGGTCCTG-3´
Q0186	-	-	5´-GGACCAAGTATGCCACCATTTTGTA-3´

Appendix C

Recombinant PfEMP1 proteins used in this study

Genome	PfEMP1	Domain sub-type	Gene source
3D7	PF11_05213/PF3D7_1150400	DBLb3_D4	
BM048	JF712902	DBLb3_D4	
BM066	JF712903	DBLb3_D4	
BM021	JF712900	DBLb3_D4	
BM057	JN037695	DBLb3_D4	
3D7	PFD1235w/PF3D7_0425800	DBLb3_D4	
MN35	KJ866957	DBLb3_D4	
HB3	VAR03	DBLb3_D4	
Dd2	VAR32/KOB85388	DBLb1_D4	
MN56	KM364031	DBLb1_D4	
A4395	KJ866958	DBLb3	
1914	AFJ66668	DBLb1_D4	
BM028	JF712901	DBLb3_D4	
-	KM364033	DBLb3	
MN062	KF984156	DBLb1_D4	
	CDO62031		Synthetic gene (https://www.eurofinsgenomics.eu/)
	CDO61797		Synthetic gene (https://www.eurofinsgenomics.eu/)
	CDO63496		Synthetic gene (https://www.eurofinsgenomics.eu/)
Dd2	VAR25	DBLb11_D4	
HB3	VAR1CSA	DBLb11_D4	
3D7	PF13_0003	DBLb9_D8	
A4393	KJ866959	DBLb3	
IT4	IT4VAR13/ABM88750	DBLb3_D4	
1983	JQ691647	DBLb3_D4	
MN35	KM364034	DBLb6	
Dd2	VAR52	DBLb7_D4	
HB3	VAR01	DBLb7_D4	
1983	JQ691649	DBLb6_D4	
IT4	IT4VAR16/AAS89259	DBLb5_D4	

Appendix D



Levels of inflammation and endothelial activation markers and age dependency in malaria patients. Symbols indicate individual soluble levels in the different age categories. Multiple variables were analysed using the Kruskal-Wallis Rank Sum Test with Dunn's Multiple Comparison Test. SM= severe malaria; UM= uncomplicated malaria

Appendix E

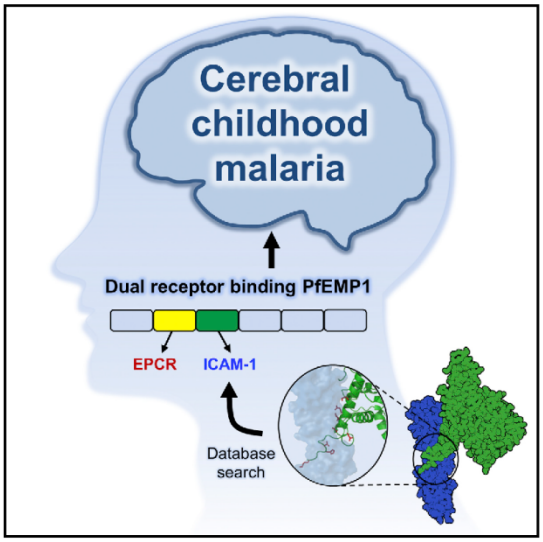
Publications

Article

Cell Host & Microbe

Structure-Guided Identification of a Family of Dual Receptor-Binding PfEMP1 that Is Associated with Cerebral Malaria

Graphical Abstract



Authors

Frank Lennartz, Yvonne Adams, Anja Bengtsson, ..., Lars Hviid, Matthew K. Higgins, Anja T.R. Jensen

Correspondence

matthew.higgins@bioch.ox.ac.uk (M.K.H.), atrj@sund.ku.dk (A.T.R.J.)

In Brief

Plasmodium falciparum-infected erythrocytes display PfEMP1 proteins that bind various endothelial receptors, including ICAM-1. Lennartz et al. structurally characterize PfEMP1 binding to ICAM-1, allowing them to identify a PfEMP1 family that simultaneously binds to both ICAM-1 and EPCR. Dual-binding PfEMP1s display stronger endothelial adhesion and are associated with cerebral malaria.

Highlights

- Structural basis for *P. falciparum* PfEMP1 binding to endothelial receptor ICAM-1defined
- A sequence motif derived from structure predicts group A PfEMP1 binding to ICAM-1
- These ICAM-1-binding PfEMP1s also all bind to endothelial protein C receptor (EPCR)
- Expression of dual ICAM-1- and EPCR-binding PfEMP1 is associated with cerebral malaria

Accession Numbers

KF984156
KJ866957
KJ866958
KJ866959
KM364031
KM364032
KM364033
KM364034
5MZA

Lennartz et al., 2017, Cell Host & Microbe *21*, 403–414
March 8, 2017 © 2017 The Author(s). Published by Elsevier Inc.
http://dx.doi.org/10.1016/j.chom.2017.02.009

CellPress

Cell Host & Microbe
Article

Structure-Guided Identification of a Family of Dual Receptor-Binding PfEMP1 that Is Associated with Cerebral Malaria

Frank Lennartz,[1,9] Yvonne Adams,[2,3,9] Anja Bengtsson,[2,3,9] Rebecca W. Olsen,[2,3] Louise Turner,[2,3] Nicaise T. Ndam,[4,5] Gertrude Ecklu-Mensah,[2,3,6] Azizath Moussiliou,[5] Michael F. Ofori,[6] Benoit Gamain,[7] John P. Lusingu,[8] Jens E.V. Petersen,[2,3] Christian W. Wang,[2,3] Sofia Nunes-Silva,[7] Jakob S. Jespersen,[2,3] Clinton K.Y. Lau,[1] Thor G. Theander,[2,3] Thomas Lavstsen,[2,3] Lars Hviid,[2,3] Matthew K. Higgins,[1,10,*] and Anja T.R. Jensen[2,3,*]

[1]Department of Biochemistry, University of Oxford, South Parks Road, OX1 3QU Oxford, UK
[2]Centre for Medical Parasitology, Department of Immunology and Microbiology (ISIM), Faculty of Health and Medical Sciences, University of Copenhagen, 1165 Copenhagen, Denmark
[3]Department of Infectious Diseases, Copenhagen University Hospital (Rigshospitalet), 2100 Copenhagen, Denmark
[4]Faculté de Pharmacie, Institut de Recherche pour le Développement (IRD), COMUE Sorbonne Paris Cité, 75013 Paris, France
[5]Faculté des Sciences de la Santé (FSS), Université d'Abomey Calavi, 01 BP 526 Cotonou, Benin
[6]Department of Immunology, Noguchi Memorial Institute for Medical Research, University of Ghana, Legon, Ghana
[7]UMR_S1134, Université Sorbonne Paris Cité, Université Paris Diderot, Inserm, INTS, Unité Biologie Intégrée du Globule Rouge, Laboratoire d'Excellence GR-Ex, 75013 Paris, France
[8]National Institute for Medical Research, Tanga Centre, 11101 Dar es Salaam, Tanzania
[9]Co-first author
[10]Lead Contact
*Correspondence: matthew.higgins@bioch.ox.ac.uk (M.K.H.), atrj@sund.ku.dk (A.T.R.J.)
http://dx.doi.org/10.1016/j.chom.2017.02.009

SUMMARY

Cerebral malaria is a deadly outcome of infection by *Plasmodium falciparum*, occurring when parasite-infected erythrocytes accumulate in the brain. These erythrocytes display parasite proteins of the PfEMP1 family that bind various endothelial receptors. Despite the importance of cerebral malaria, a binding phenotype linked to its symptoms has not been identified. Here, we used structural biology to determine how a group of PfEMP1 proteins interacts with intercellular adhesion molecule 1 (ICAM-1), allowing us to predict binders from a specific sequence motif alone. Analysis of multiple *Plasmodium falciparum* genomes showed that ICAM-1-binding PfEMP1s also interact with endothelial protein C receptor (EPCR), allowing infected erythrocytes to synergistically bind both receptors. Expression of these PfEMP1s, predicted to bind both ICAM-1 and EPCR, is associated with increased risk of developing cerebral malaria. This study therefore reveals an important PfEMP1-binding phenotype that could be targeted as part of a strategy to prevent cerebral malaria.

INTRODUCTION

Malaria affects hundreds of millions of people each year. While most cases are not life threatening, a significant number of infections by *Plasmodium falciparum* result in severe malaria, manifested in one or more of three major syndromes: anemia, respiratory distress, and cerebral malaria (CM). These occur as parasites infect and divide within human erythrocytes. The infected erythrocytes adhere to blood vessels and tissue surfaces, allowing the parasite to avoid clearance by the spleen. In addition, organ-specific sequestration can have major consequences for development of specific malaria symptoms, particularly in CM, the most debilitating form of the disease. Here, infected erythrocytes accumulate in the brain, occluding blood flow, inducing inflammation, and leading to major neurological complications (Hviid and Jensen, 2015). Even with antimalarial treatment, mortality rates due to CM in children range between 15% and 20% (Dondorp et al., 2010; Seydel et al., 2015), and survivors of CM often suffer from a wide variety of long-lasting neurological damage, which can result in loss of motor function, impairment in learning and language capability, or an increased risk of epilepsy (Birbeck et al., 2010; Idro et al., 2005).

During blood stage infection, the parasite expresses members of different variant surface protein families. Of these, *Plasmodium falciparum* erythrocyte membrane proteins 1 (PfEMP1) are best understood. They are displayed on infected erythrocyte surfaces and tether the cells to various receptors. Each *Plasmodium falciparum* genome contains ~60 PfEMP1-encoding *var* genes, which can be classified according to their chromosomal context into groups A–E. They have large ectodomains containing multiple duffy binding-like (DBL) and cysteine-rich inter-domain region (CIDR) domains that can interact with specific human endothelial receptors. Sequence analysis allows the CIDR and DBL domains to be divided into general subgroups associated with specific binding phenotypes (Hviid and Jensen, 2015). In particular, subclasses of DBLβ domains found in group A, B, and C PfEMP1 bind ICAM-1 (Bengtsson et al., 2013; Howell et al., 2008; Janes et al., 2011); CIDRα1 domains from group A PfEMP1 bind endothelial protein C receptor (EPCR) (Lau et al., 2015; Turner et al., 2013);

and group B and C PfEMP1 contain CIDRα2–6 domains, which bind CD36 (Hsieh et al., 2016; Robinson et al., 2003).

With a number of receptors available to bind to PfEMP1, a major goal has been to determine whether PfEMP1s with specific receptor-binding phenotypes are associated with cerebral and other forms of severe malaria. These studies mostly focused on children, as adults from malaria endemic areas have a lower risk of death from complications during infection. Early studies showed that parasites that express group A PfEMP1 or those that form rosettes are linked with severe malaria (Doumbo et al., 2009; Jensen et al., 2004). The search was further focused with the discovery that severe malaria is associated with a subset of group A and B/A PfEMP1s (Avril et al., 2012; Claessens et al., 2012; Lavstsen et al., 2012), which were later shown to bind to EPCR (Turner et al., 2013). This demonstrated a link between severe malaria and expression of EPCR-binding PfEMP1 (Bernabeu et al., 2016; Jespersen et al., 2016; Turner et al., 2013).

While these studies established associations between severe malaria and particular groups of PfEMP1, no connections have been identified to any specific individual severe malaria syndrome. In particular, attempts to link CM to expression of certain groups of PfEMP1 remain inconclusive. Some studies have correlated cerebral disease with ICAM-1 binding (Ochola et al., 2011; Silamut et al., 1999; Turner et al., 1994), but others found no such link (Newbold et al., 1997; Rogerson et al., 1999). Indeed, ICAM-1-binding DBLβ domains occur in B- and C-type PfEMP1s that are associated with uncomplicated malaria as well as A-type PfEMP1s associated with severe disease, suggesting that ICAM-1 binding alone is not a driver of CM (Bengtsson et al., 2013; Howell et al., 2008; Janes et al., 2011).

A significant obstacle to associating specific adhesion phenotypes with disease outcomes has been the inability to directly predict, using sequence information, adhesion traits of PfEMP1 expressed in patients. While EPCR- and CD36-binding CIDR domains can be predicted from their sequences (Hsieh et al., 2016; Robinson et al., 2003; Turner et al., 2013), ICAM-1-binding domains cannot. We therefore aimed to understand the molecular basis for ICAM-1 binding by A-type PfEMP1, to define a motif allowing the identification of ICAM-1-binding DBLβ domains from sequence alone, and to determine whether the expression of these ICAM-1-binding domains is associated with the development of CM.

RESULTS

The Structural Basis for ICAM-1 Binding by PfEMP1

In the absence of a structure of a DBLβ domain bound to ICAM-1, we purified complexes of a diverse set of DBLβ domains from group A PfEMP1 bound to the N-terminal two domains of ICAM-1 (ICAM-1^{D1D2}), increasing the likelihood of obtaining well-diffracting crystals. Of these, a complex of the PF11_0521 (PlasmoDB: PF3D7_1150400) DBLβ3_D4 domain and ICAM-1^{D1D2} formed crystals that diffracted to 2.8 Å resolution (Table S1). We used molecular replacement, with previous structures of ICAM-1 domains and a model derived from the varO DBLα domain as search models, to determine the structure (Figures 1A and 1B). We also used small-angle X-ray scattering to confirm that the arrangement of the complex was the same in solution as that observed in the crystals, and that it further matched the arrangement of a complex of the DBLβ domain bound to full-length ICAM-1^{D1D5} (Figure S1).

The PF11_0521 DBLβ3_D4 domain adopts the classical DBL domain fold, consisting of a core of α helices, decorated by numerous loops (Figures 1, S2A, and S2B). In contrast to previously characterized DBL domains, an extended α helix and a glycine and proline-rich linker form a unique feature that protrudes from the domain and generates part of the ICAM-1 interaction site (Figures 1, S2A, and S2B). Indeed, a chimeric protein containing this region of an ICAM-1-binding domain, transplanted to a related, non-binding DBLβ domain, produced an ICAM-1-binding domain (Figure 2A), and antibodies targeting this region (either purified from peptide-immunized rat or from human plasma) prevented infected erythrocytes from binding to ICAM-1 (Figures 2B and 2C).

The DBLβ domain interacts with ICAM-1 through a complex, elongated binding interface that contains three distinct subsites with a total surface area of 1,144 $Å^2$ (Figure 1C). Site 1 is formed from a patch of hydrophobic residues along one side of the extended α helix of DBLβ that interacts with a corresponding hydrophobic patch on the surface of ICAM-1 D1. Site 2 is formed by the subsequent linker of the DBLβ domain and forms hydrogen bonds with both ICAM-1 D1 and D2 domains. Site 3 is formed from part of a long loop that protrudes from the DBLβ domain and also contacts both ICAM-1 D1 and D2. The parts of this loop not in contact with ICAM-1 are not observed in the electron density, indicating flexibility (Figures 1B and S2C). This loop is indeed predicted to be intrinsically disordered (Figure S2D), but becomes partially ordered when bound to ICAM-1. Such a flexible region of a protein, which forms part of, or is immediately adjacent to, its ligand-binding site, is an immune evasion strategy used by surface proteins from other pathogens (Kim et al., 2004; Kwong et al., 1998), but had not been previously observed in any PfEMP1 domain bound to its receptor.

A Sequence Motif Allows for the Prediction of ICAM-1 Binding A-type PfEMP1

We next aimed to use the crystal structure to identify key molecular determinants used by the DBLβ domains for ICAM-1 binding, and to thereby identify a sequence motif that can be used to predict these ICAM-1 binders. For this, we mutated residues in the DBLβ domain that either directly contact ICAM-1 or are responsible for the unusual architecture of the binding site (Figure 2D; Table S2). By testing the eight residues that directly interact with ICAM-1 (Figure S3; Table S2), we found that each of the three subsites contains a critical residue that, when mutated, disrupts ICAM-1 binding (Figure 2E; Table S3). In addition, certain structural residues, in particular glycines and prolines, are essential for ICAM-1 binding as they allow sharp backbone bends that correctly present binding residues (Figures 2 and S3; Table S3). To ensure that the observed effects were not due to disruption in the overall fold of the DBL domain, all mutants that showed an effect on ICAM-1 binding were tested by circular dichroism spectroscopy and found to be indistinguishable from the wild-type domain (Figure S4).

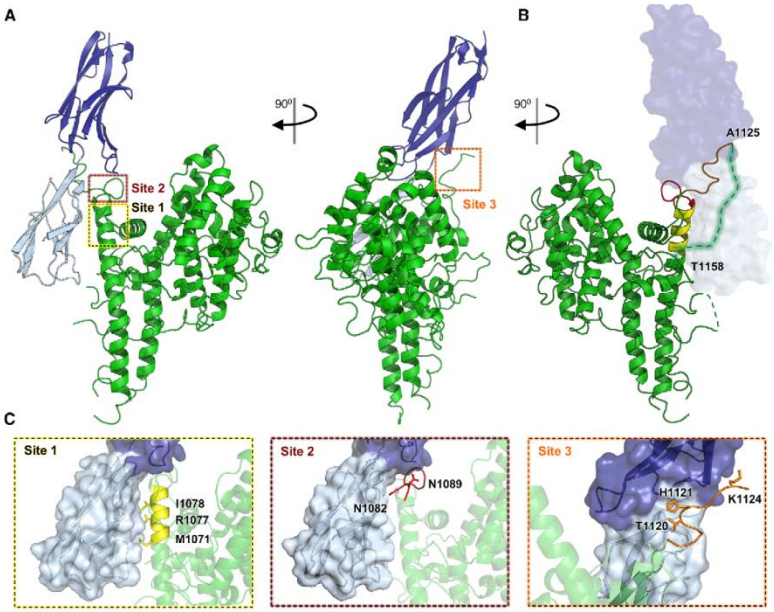


Figure 1. The Structural Basis for ICAM-1 Binding by Group A PfEMP1s

(A) Front and side views of the PF11_0521 DBLβ_D4 domain (green) bound to ICAM-1^{D1D2} (D1, light blue; D2, dark blue). Dashed boxes highlight three sites that make direct contact with ICAM-1.

(B) Back view of PF11_0521 DBLβ_D4 domain (green) bound to ICAM-1^{D1D2}. Dashed lines represent residues not visible in the electron density map with the disordered regions near site 3 (orange) of the ICAM-1-binding site highlighted.

(C) Three distinct sites in the PF11_0521 DBLβ_D4 domain directly interact with ICAM-1 with residues that mediate this interaction indicated.

See also Figures S1 and S2 and Tables S1 and S2.

Based on this analysis, coupled with sequence analysis of previously identified A-type ICAM-1 binding domains (Bengtsson et al., 2013), we defined a sequence motif ([I/V/L]x_3N[E]GG[P/A] x$Y_{x_{27}}$GPPx_3H) that contains the determinants for ICAM-1 binding by these group A PfEMP1s. This motif was used to search sequence databases, identifying a total of 145 ICAM-1-binding DBL domains (mostly A-type PfEMP1s with a few B/A-type PfEMP1s) that contain the motif and are therefore predicted to bind to ICAM-1 (Table S4). To test these predictions, we used ELISA to analyze the interaction between ICAM-1 and 16 motif-containing DBLβ domains, selected to represent sequence diversity (Table S4). All 16 DBLβ domains with the motif bound ICAM-1, while 10 group A DBLβ domains without the motif did not (Figures 2F and 3A; Table S4). In sequence analysis of DBLβ domains (n = 55) with known ICAM-1-binding properties and DBLβ S3 sequences (n = 1,823) from whole-genome studies, the ICAM-1-binding group A and B/A domains with the motif clustered separately from other domains, including group B and C ICAM-1-binding DBLβ domains lacking the motif (Figures 3 and S5). Taken together, this suggests at least two groups of ICAM-1-binding PfEMP1s with different evolutionary histories and that the majority of ICAM-1-binding group A and B/A PfEMP1s can be predicted from the presence of a highly conserved binding site (Figures 2G and 2H).

Parasites Expressing ICAM-1-Binding DBLβ Domains Elicit Inhibitory Antibodies and Are Associated with CM

The high sequence identity of DBLβ domains containing the motif (Table S4) encouraged us to determine whether these domains might induce cross-reactive antibodies during natural infections. We therefore screened samples from Liberian adults for the presence of immune plasma that inhibited DBLβ domains from binding to ICAM-1. Plasma IgG from a pool of these inhibitory samples was then affinity purified separately on three different DBLβ domains containing the motif (Dd2var32, KM364031, and PFD1235w [PlasmoDB: PF3D7_0425800] DBLβ_D4) and tested for the ability to inhibit ICAM-1 binding of DBLβ domains (Figure 4A). The IgG showed the potential to broadly inhibit ICAM-1 binding by a panel of motif-containing DBLβ domains from group A PfEMP1, but did not prevent ICAM-1 binding by

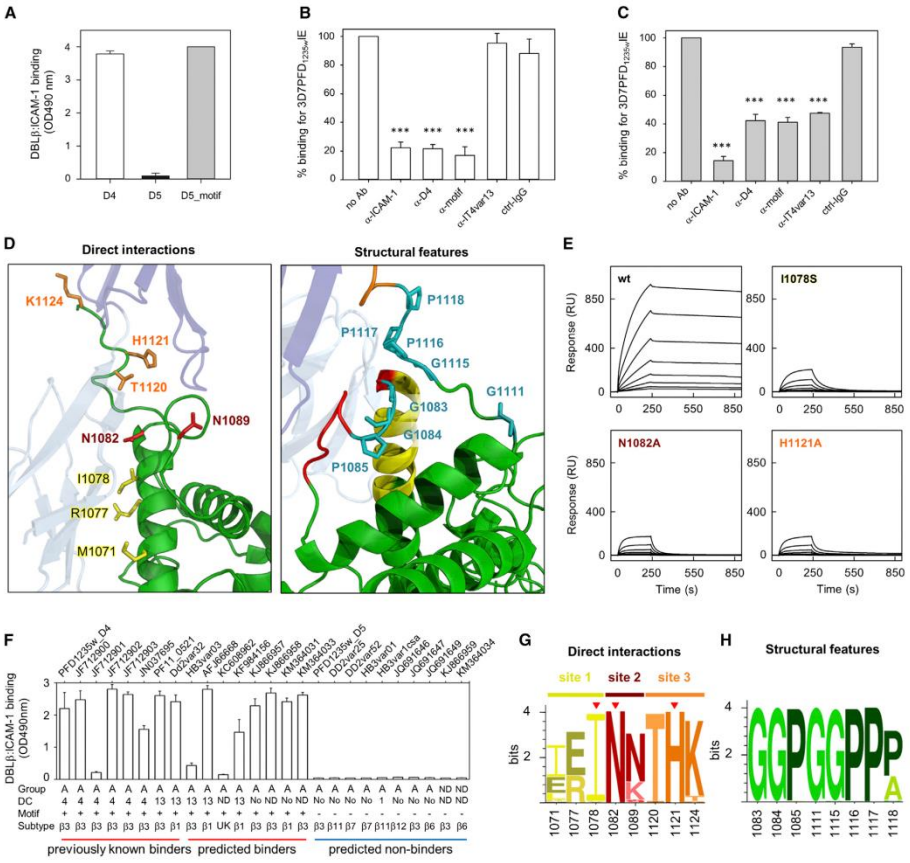


Figure 2. Structure-Guided Identification of a Motif that Predicts ICAM-1 Binding

(A) ICAM-1 binding (ELISA OD 490 nm; ± SD) of recombinant 3D7 PFD1235w DBLβ3_D4, PFD1235w DBLβ3_D5, and a chimeric DBLβ3_D5 containing the ICAM-1-binding motif region of DBLβ3_D4 (D5_motif).

(B and C) Inhibition of 3D7 PFD1235W infected erythrocyte adhering to ICAM-1 under flow in the presence of anti-ICAM-1 Abs, affinity-purified anti-PFD1235w DBLβ3_D4 (anti-D4) IgG, anti-PFD1235w_motif IgG, anti-ITVAR13, and control IgG. IgG antibodies affinity purified from (B) rat anti-serum and (C) human serum. ± SD of a minimum of three independent experiments done in triplicate. Data were analyzed using one-way ANOVA. Asterisks indicate significance ($p = 0.0004$).

(D) The three sites within the PF11_0521 DBLβ3_D4-binding site, indicating residues that directly contact ICAM-1 (site 1, yellow; site 2, red; site 3, orange). Structural residues important for positioning of interacting residues are highlighted in teal.

(E) Surface plasmon resonance curves for injection of 2-fold dilution series of DBLβ wild-type and binding site mutants over ICAM-1^{D1D5}.

(F) ICAM-1 binding (ELISA OD 490 nm; ± SD three replicates) of 26 recombinant group A DBLβ domains (4 DBLβ1, 14 DBLβ3, 2 DBLβ6, 2 DBLβ7, 2 DBLβ11, 1 DBLβ12, and 1 DBLβ unknown sub-class), confirming prediction of binding domains. "DC" indicates in which domain cassette the domain is found. "ND" indicates that the DC type is unknown as only the DBLβ sequence is available. "No" indicates that the domain is not part of a known DC. Presence of DC13 prior to DBLβ is indicated.

(G and H) Sequence logo showing conservation of (G) residues that contact ICAM-1 and (H) residues important for the unusual architecture of the ICAM-1-binding site, based on 145 DBLβ domains predicted to bind ICAM-1. Red triangles, residues critical for direct interaction with ICAM-1.

See also Figures S3–S5 and Tables S2, S3, and S4.

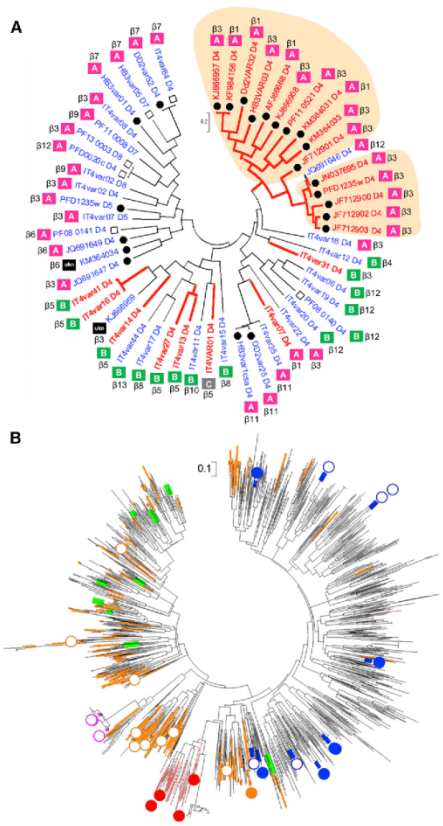


Figure 3. Phylogeny of ICAM-1- and Non-binding DBLβ Domains

(A) Maximum likelihood tree of 55 complete DBLβ domains. The tree is drawn to scale, with branch lengths measured in substitutions per site. Red, ICAM-1 binders; blue, ICAM-1 non-binders (Avril et al., 2016; Bengtsson et al., 2013; Janes et al., 2011; Lau et al., 2015). Filled circles, DBLβ domains tested in this study; open squares, DBLβ domains tested, but data not shown. UKN indicates unknown group ID. Shaded areas indicate DBLβ with the ICAM-1 motif.

(B) Maximum likelihood tree of 1,823 complete DBLβ S3 sequences from the seven published genomes and 226 annotated Sanger genomes. Filled circles, DBLβ experimentally shown as ICAM-1 binders. Open circles, DBLβ experimentally shown as ICAM-1 non-binders. Colors indicate the CIDR domain N-terminal to DBLβ: red, EPCR binders with ICAM-1-binding motif; orange, EPCR binders with no ICAM-1-binding motif; green, non-EPCR binders (group A); blue, CD36 binders; magenta, VAR1.

See also Figure S5 and Table S4.

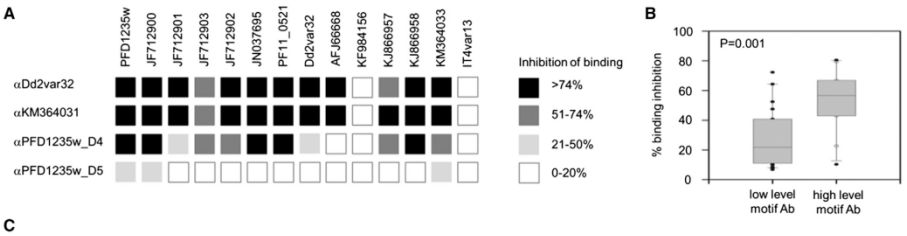
a DBLβ domain from a group B PfEMP1 (IT4VAR13). In contrast, IgG that was affinity purified on a closely related non-ICAM-1-binding DBLβ domain that lacks the motif (PFD1235w DBLβ_D5) did not significantly inhibit binding of any DBLβ domain tested (Figure 4A). Therefore, plasma from adults in a malaria endemic region contains IgG that shows the potential to broadly inhibit ICAM-1 binding of motif-containing PfEMP1.

We also tested whether children develop antibodies that specifically target the ICAM-1 binding site of the motif-containing DBLβ domains. To this end, we screened plasma samples from *Plasmodium falciparum*-exposed non-hospitalized Tanzanian children living in an area of high malaria transmission against a peptide containing the motif. A high proportion (69 of 76) of samples contained motif-reactive antibodies, and the DBLβ:ICAM-1-binding inhibition was significantly higher in plasma with high reactivity (ELISA optical density [OD] > 1) against the motif when compared with those with low motif reactivity (ELISA OD < 1) (Figure 4B).

Next, to correlate the expression of the group A ICAM-1-binding PfEMP1 with disease outcomes, we used the sequence motif to design primers to selectively amplify motif-encoding *var* gene transcripts. We used these to assess transcript levels in children with CM and severe malarial anemia (SA), and in other pediatric malaria patients (other malaria, OM; non-CM and non-SA) (Figure 4C; Table S5). The abundance of *var* gene transcripts was calculated relative to the transcript of an endogenous control gene and was translated into transcript units (Lavstsen et al., 2012). This analysis showed higher transcription of motif-encoding, ICAM-1-binding PfEMP1 in children with CM than in children with SA or in those admitted to hospital with malaria without these signs of severity (OM). Due to the challenges in acquiring a sufficiently large number of samples from single locations, patients were recruited at different sites and logistic regression modeling was used to correct for this. Comparison of the three groups showed that levels of transcripts encoding ICAM-1-binding PfEMP1 were significantly associated with risk of CM ($p = 0.02$) when comparing CM patients to malaria patients without CM (SA and OM). In contrast, transcript levels reported by primers targeting loci encoding EPCR-binding PfEMP1 (Mkumbaye et al., 2017) was not higher in CM patients compared to SA plus OM patients ($p = 0.751$). These data suggest a direct link between CM and expression of this particular subset of ICAM-1-binding PfEMP1s (Figure 4C).

Motif-Containing ICAM-1-Binding DBLβ Domains Are Associated with EPCR-Binding Domains, Allowing Simultaneous Dual Receptor Binding

The correlation between CM and expression of group A PfEMP1 containing the conserved ICAM-1-binding site raised the question of why ICAM-1 binding was not unambiguously associated with CM in previous studies. With ICAM-1 binding alone unlikely to be the driver of cerebral disease, we therefore examined the broader context of the motif-containing ICAM-1-binding domains. We made the striking observation that all tested PfEMP1s containing these domains also contain a neighboring EPCR-binding CIDRα1 domain (Table S4; Figures 3B and S5). These PfEMP1s form a separate cluster from PfEMP1s that contain an EPCR-binding domain, but no motif containing ICAM-1-binding domain (Figures 3B and S5), and we find that 14% of all EPCR binders have such a motif-containing ICAM-1-binding domain. In contrast to this, B- and C-type ICAM-1-binding PfEMP1s are frequently associated with CIDRα domains that



C

Predicted binding phenotype	Cerebral malaria (CM) *(n= 53)*	Severe anemia (SA) *(n= 27)*	Other malaria (OM) *(n= 36)*	CM vs. SA+OM, *P-value*
ICAM-1 binding Gr A DBLβ	7.5 [1.4; 32.9]	3.0 [1.0; 8.9]	2.4 [1; 12.3]	0.020
EPCR binding CIDRα	82.7 [36.4; 171.3]	46.4 [22.7; 126.2]	77.0 [29.9; 154.3]	0.751

Figure 4. The Conserved ICAM-1 Binding Site Is Recognized by Patient Plasma and Linked to CM

(A) The inhibition of DBLβ domains binding to ICAM-1 by IgG from a plasma pool from Liberian *P. falciparum*-exposed adults purified on ICAM-1-binding DBLβ_D4 domain from Dd2var32, KM364031, and PFD1235w or on a closely related, but non-ICAM-1-binding DBLβ_D5 domain from PFD1235w. ICAM-1 inhibitory capacity is >74% (black), 51%–74% (dark gray), 21%–50% (light gray), and 0%–20% (white).

(B) ICAM-1-binding inhibition by plasma with low (ELISA OD < 1) and high (ELISA OD > 1) anti-motif-DBLβ IgG in *P. falciparum*-exposed Tanzanian children (1–17 years) (Lusingu et al., 2004). Boxplot with median. Whiskers, 5% and 95% percentiles.

(C) Transcript levels (in arbitrary transcription units, Tu) of *var* gene subtypes in Ghanaian, Tanzanian, and Beninese children hospitalized with malaria, shown with medians and 25% and 75% percentiles.

See also Table S5.

interact with CD36 and are distributed across the phylogenetic tree (Figure 3B; Table S4). This suggests that the group A ICAM-1-binding PfEMP1s are a subset of the ICAM-1-binding PfEMP1s, separate from group B and C PfEMP1s, and that they also have the capacity to bind to EPCR.

The link between the presence of both ICAM-1- and EPCR-binding domains within a single PfEMP1 raised the intriguing possibility that these PfEMP1s might simultaneously engage both receptors. A few PfEMP1s have previously been identified that contain both EPCR-binding CIDRα domains and ICAM-1-binding DBLβ domains (Avril et al., 2016; Oleinikov et al., 2009; Turner et al., 2013). However, it was unknown whether they could interact with both receptors simultaneously, and whether such dual binding would have consequences for infected erythrocyte adhesion. We therefore produced a set of three different recombinant PfEMP1s and used surface plasmon resonance to test their potential for binding to the two receptors simultaneously. Indeed, we found that protein constructs containing the ICAM-1- and EPCR-binding domains of these PfEMP1s all bound simultaneously to ICAM-1 and EPCR with nanomolar affinity (Figures 5 and S6; Table S6).

Next, we examined the effect of dual binding to ICAM-1 and EPCR in the context of infected erythrocytes. For this, erythrocytes infected with parasites expressing PfEMP1 (PFD1235w or HB3VAR03) predicted to bind both receptors were selected in vitro (Figure S7) and binding to both receptors was tested under flow conditions using recombinant receptors (Figures 6A–6C) and in an immunofluorescence assay (Figures 6G–6I). These studies were performed at a range of flow rates from 0.5 to 5.0 dyn/cm^2, chosen to induce the most physiologically relevant shear stresses. Infected erythrocytes were capable of attaching between 0.5 and 1.0 dyn/cm^2 in the presence of either recombinant receptor alone. When ICAM-1 and EPCR were simultaneously available at comparable molar concentrations, erythrocytes infected with parasites expressing dual-binding PfEMP1 demonstrated an increased ability to bind at a higher shear stress, i.e., 2 dyn/cm^2, compared to when either receptor was available alone, though this adhesion did not exceed that measured at 1 dyn/cm^2 (Figures 6A and 6B; Table S7A). In contrast, erythrocytes infected with parasites known to bind ICAM-1 and CD36 (IT4VAR13; Figures S7E and S7F) showed increased adhesion in the presence of both ICAM-1 and CD36 compared to CD36 binding alone at lower shear stress, but did not confer the ability to withstand higher shear stresses, and the adhesion decreased in response to increasing shear stress (Figure 6C). The final parasite, known to bind to PECAM-1 (PF11_0008 [PlasmoDB: PF3D7_1100200]; Figures S7G and S7H), failed to bind any of the receptors tested (Figure S7L).

To further test the physiological relevance of our observations, we assessed the binding of infected erythrocytes to HBMEC with and without tumor necrosis factor α (TNFα) treatment (Figures 6D–6F and S7I–S7K). To determine the contribution of individual receptors to binding, antibodies against either ICAM-1 or EPCR alone or a combination of both were used. With HB3VAR03, at 0.5 dyn/cm^2 the greatest inhibition was observed on cells treated with both anti-receptor antibodies simultaneously (80%, p = 0.0361; Figure 6D), while HBMEC treated with either anti-ICAM-1 or anti-EPCR antibodies demonstrated a non-significant reduction in adhesion (Table S7B). A similar effect was observed at 0.75 dyn/cm^2. With increasing shear stress,

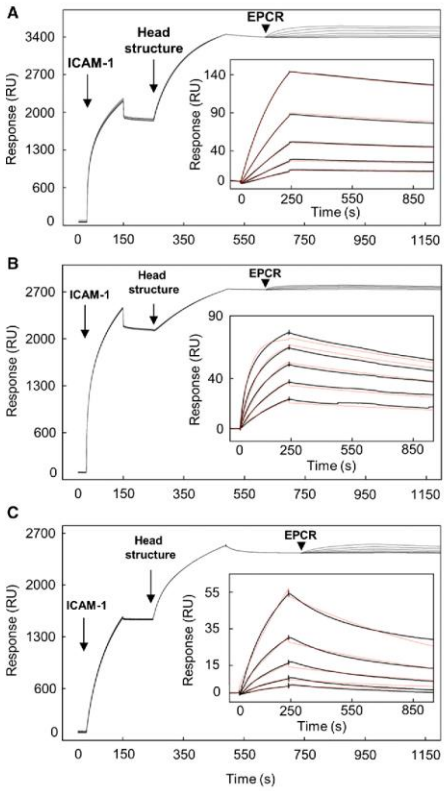


Figure 5. PfEMP1 Can Simultaneously Bind to Both ICAM-1 and EPCR

ICAM-1^{D1D5} was immobilized at fixed concentration, followed by injection of the respective head structure, (A) PF11_0521 DBLα1.7-CIDRα1.4-DBLβ3, (B) Dd2var32 DBLα1.7-CIDRα1.4-DBLβ3, and (C) PFD1235w CIDRα1.6-DBLβ3, also at fixed concentration. A 2-fold dilution series of EPCR was injected over the resulting ICAM-1::head structure complex. Arrows show start points of protein injection. The insets show data fitted to a one-site kinetic model for binding of EPCR to the respective ICAM-1::head structure complex. Data (black lines) are modeled to a one-site model (red lines). See also Figure S6 and Table S6.

the activity of single-antibody treatment was enhanced, and at 1 dyn/cm^2 this adhesion inhibition was significant for each individual anti-receptor antibody (Figure 6D; Table S7B), showing that the ability of HB3VAR03 to bind to both receptors is important at higher shear stresses. In comparison, 3D7PFD1235w infected erythrocytes were significantly inhibited by the presence of either antibody at all shear stresses tested (Figure 6E; Table S7B). As expected, IT4VAR13 only bound ICAM-1 and not EPCR expressed on the surface of HBMEC (Figure 6F). When the same experiments were conducted on activated cells, all three parasite lines showed increased reliance on ICAM-1 for adhesion, with HB3VAR03- and 3D7PFD1235w-infected erythrocytes switching to a single-receptor phenotype due to down-regulation of EPCR expression upon cell activation by TNFα (Menschikowski et al., 2009) (Figures S7I–S7K). In summary, the ability to bind to both ICAM-1 and EPCR gives parasites significantly greater versatility, allowing for an increased capacity to adhere at physiological higher shear stresses, and enables them to continue to adhere despite changes in receptor expression upon cell activation.

Association of ICAM-1 and EPCR Dual-Binding PfEMP1 with the Development of CM

Our ability to predict dual-binding PfEMP1 from sequence next allowed us to assess the link between the presence of parasites expressing such PfEMP1 and the development of different severe malaria syndromes in patient isolates, and to compare this with the disease outcomes associated with PfEMP1s that bind EPCR alone. For this, we analyzed a dataset containing the full sequences of the six most abundant PfEMP1 transcripts expressed in 45 children hospitalized with malaria (on average corresponding to 75% of total *var* transcripts; see Supplemental Experimental Procedures), the biggest available dataset of its kind (Jespersen et al., 2016). These children presented with CM or SA, or were admitted to hospital with malaria, but without these signs of severity, and were immediately treated with anti-malarial drugs (Table S5). We identified dual-binding PfEMP1 from the sequence data by presence of a DBLβ domain containing the group A ICAM-1-binding motif adjacent to an EPCR-binding CIDRα1 domain. EPCR-binding PfEMP1s were identified by the presence of CIDRα1 domains alone. In this dataset, parasites expressing PfEMP1 predicted to bind EPCR, but not ICAM-1, were more likely to be found in severe malaria cases (CM plus SA) ($p = 0.040$) (Figure 7B). However, there was no association between EPCR binding and either of the specific severe malaria syndromes (with $p = 1.000$ for comparison of CM with all other types of malaria). In contrast, parasites expressing dual ICAM-1- and EPCR-binding PfEMP1 were specifically ($p = 0.016$) associated with CM, when compared with non-cerebral disease (SA plus OM) (Figure 7A). These data support our finding that the expression of ICAM-1-binding DBLβ domains containing the motif, which to date has been 100% associated with dual ICAM-1- and EPCR-binding PfEMP1 (Figures 3 and S5; Table S4), is associated with the development of cerebral symptoms (Figures 4C and 7). Therefore, while expression of PfEMP1s that bind to EPCR alone is not linked to any specific severe malaria syndrome, the presence of dual-binding motif-containing PfEMP1s is associated with the development of CM.

DISCUSSION

A major challenge in associating malaria disease phenotypes to the expression of PfEMP1s with specific binding properties has been the ability to predict ligand-binding capability from sequence alone. In this study, we used structural studies to define a sequence motif that allowed the identification of a large set of

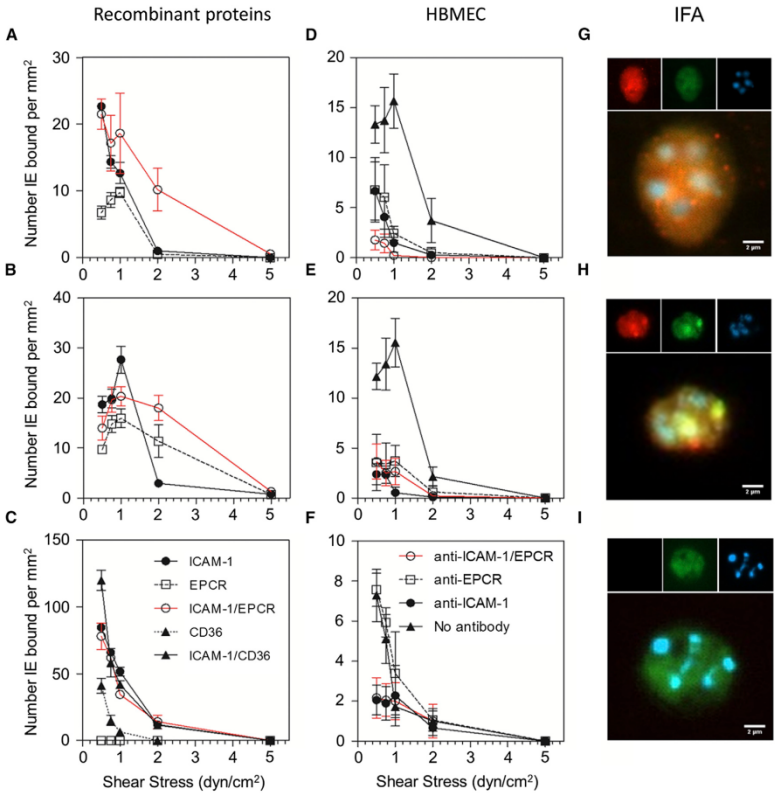


Figure 6. Binding to ICAM-1 and EPCR Increases Adhesion of Infected Erythrocytes

(A–F) Erythrocytes infected with (A and D) HB3VAR03 (predicted ICAM-1 and EPCR binder), (B and E) 3D7PFD1235w (predicted ICAM-1 and EPCR binder), and (C and F) IT4VAR13 (ICAM-1 and CD36 binder) binding to (A–C) receptor-coated microslides under flow conditions using recombinant ICAM-1, rEPCR, rICAM-1, and rEPCR; rCD36; or rICAM-1 and rCD36, and to (D–F) resting human brain microvascular endothelial cells (HBMEC). To demonstrate specific adhesion, channels coated with HBMEC were pre-incubated with anti-ICAM-1 (40 μg/mL), anti-EPCR (10 μg/mL), or anti-ICAM1 and EPCR combined (40 and 10 μg/mL, respectively). Flow experiments were done in parallel with the same conditions for immobilizing proteins or cell-coated chips. Values are bound infected erythrocyte per mm^2 ± SEM. Data were also analyzed by two-way ANOVA with Tukey's multiple comparison (Tables S7A and S7B).

(G–I) Immunofluorescence images of erythrocytes infected with parasite strains (G) HB3VAR03, (H) 3D7PFD1235w, and (I) IT4VAR13. Complexes of ICAM-1 or EPCR were added to measure binding by immunofluorescence. Main panels show overlays of ICAM-1 (green), EPCR (red), and nuclear (blue) staining. Inserts show single channels. Scale bar, 2 μm.

See also Figure S7 and Table S7.

ICAM-1-binding group A PfEMP1s from sequence. We also show that these ICAM-1-binding PfEMP1s all contain an additional EPCR-binding CIDRα1 domain and that this allows binding to both ligands. Finally, we show that the presence of parasites expressing PfEMP1s that bind to both ICAM-1 and EPCR is associated with increased risk of developing cerebral symptoms to *P. falciparum* infections.

This link between dual binding and CM is supported by two independent studies (Figures 4C and 7). However, while significant, it is not absolute. This may partly be because the data show a snapshot of the parasites present in the peripheral blood of the children at the time of hospital admission, and the children involved were treated with antimalarial medication immediately upon diagnosis, preventing further development of the disease.

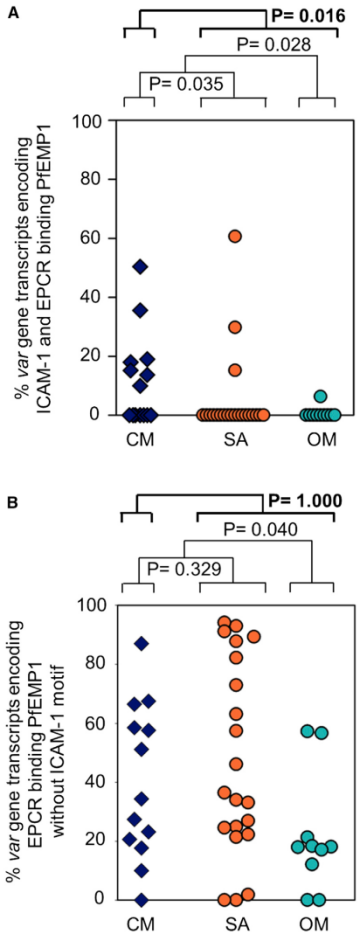


Figure 7. Dual-Binding PfEMP1s Correlate with CM

Percentages of specific *var* gene transcripts in infected erythrocytes from 45 hospitalized Tanzanian children.

(A) Percentage of *var* gene transcripts encoding PfEMP1s predicted to bind both ICAM-1 and EPCR.

(B) Percentage of transcripts that encode PfEMP1 predicted to bind EPCR and not containing the ICAM-1-binding motif. CM, cerebral malaria; SA, severe anemia; OM, children without the signs of severity found in the other groups. See also Table S5.

For example, in the analysis of whole PfEMP1 sequences (Figure 7), we cannot exclude the possibility that the small number of patients (3/21) expressing dual-binding PfEMP1 and presenting with SA, but not CM, may have developed cerebral symptoms if left untreated, causing the association detected here to be an underestimate. There are also risks in overestimating the link between CM and expression of this group of PfEMP1s due to limitations in the sample size available for each of these studies. However, this is mitigated by the fact that we observe the same association between dual-binding PfEMP1s and CM in two independent studies with samples from different patient populations from three African countries (Figures 4C and 7), suggesting that the presence of these PfEMP1s on the infected erythrocyte surface is a risk factor for the development of CM.

There are several possible reasons why dual-binding PfEMP1s might contribute to the development of CM. One consequence of dual binding is increased endothelial adhesion under conditions of high shear stress (Figure 6). CM is associated with increased sequestered parasite biomass throughout the circulatory system, and this appears to have disproportionate effects on the regulation of the homeostasis of the brain, making increased adhesion a risk factor (Cunnington et al., 2013; Moxon et al., 2009; Storm and Craig, 2014). In addition to these general effects, specific consequences for the brain may occur due to modulation of signaling mediated by ICAM-1 and EPCR, as EPCR-mediated signaling and ICAM-1 expression are linked by a complex interplay that might also contribute to cerebral disease. Infected erythrocytes prevent EPCR from interacting with its natural ligands, inhibiting signaling pathways that induce anti-inflammatory responses and protect the integrity of the endothelium (Petersen et al., 2015; Turner et al., 2013). Indeed, inflammation and brain swelling both occur in CM and are important in disease development. In addition, blockage of EPCR signaling increases expression of several PfEMP1 adhesion receptors, including ICAM-1 (Moxon et al., 2013). Paradoxically, parasite sequestration in the brain is also linked to loss of EPCR, removing an important regulator of pro-coagulation responses (Moxon et al., 2013). The capacity to bind to both receptors may give the parasite more potential to resist endothelial changes in response to binding (i.e., downregulation of EPCR) and to remain sequestered in the brain. Together with the increased expression of ICAM-1 on endothelial cells, this could lead to a vicious cycle that further exacerbates disease.

CM is a complex disease, and in addition to dual-binding PfEMP1, various other aspects of both host and parasite biology will influence the development of cerebral symptoms. Other surface protein families, including STEVORs, RIFINs, and the PfEMP1 involved in rosetting (the clustering of infected and uninfected erythrocytes), have been implicated in severe malaria (Doumbo et al., 2009; Goel et al., 2015; Niang et al., 2014; Rowe et al., 1997) and further study is required to assess whether they too have a significant impact on the development of cerebral symptoms. In addition, host genetic factors, such as erythrocyte surface protein polymorphisms, could affect the capacity of the parasite to invade erythrocytes, altering parasitemia and therefore severity (Band et al., 2015). Nevertheless, the identification of dual-binding PfEMP1 as a risk factor for CM highlights these molecules as a promising and tractable candidate for the development of a vaccine to contribute to the prevention of this form of the disease.

A major challenge in targeting PfEMP1s through vaccination is their high sequence variability, driven by a diversifying selection

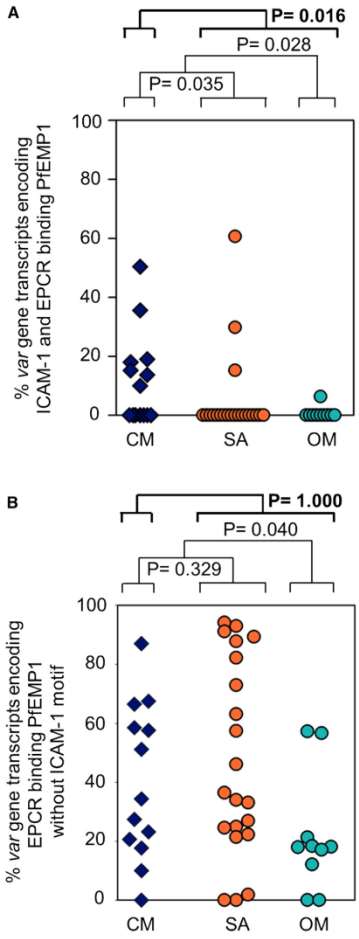


Figure 7. Dual-Binding PfEMP1s Correlate with CM

Percentages of specific *var* gene transcripts in infected erythrocytes from 45 hospitalized Tanzanian children.

(A) Percentage of *var* gene transcripts encoding PfEMP1s predicted to bind both ICAM-1 and EPCR.

(B) Percentage of transcripts that encode PfEMP1 predicted to bind EPCR and not containing the ICAM-1-binding motif. CM, cerebral malaria; SA, severe anemia; OM, children without the signs of severity found in the other groups. See also Table S5.

For example, in the analysis of whole PfEMP1 sequences (Figure 7), we cannot exclude the possibility that the small number of patients (3/21) expressing dual-binding PfEMP1 and presenting with SA, but not CM, may have developed cerebral symptoms if left untreated, causing the association detected here to be an underestimate. There are also risks in overestimating the link between CM and expression of this group of PfEMP1s due to limitations in the sample size available for each of these studies. However, this is mitigated by the fact that we observe the same association between dual-binding PfEMP1s and CM in two independent studies with samples from different patient populations from three African countries (Figures 4C and 7), suggesting that the presence of these PfEMP1s on the infected erythrocyte surface is a risk factor for the development of CM.

There are several possible reasons why dual-binding PfEMP1s might contribute to the development of CM. One consequence of dual binding is increased endothelial adhesion under conditions of high shear stress (Figure 6). CM is associated with increased sequestered parasite biomass throughout the circulatory system, and this appears to have disproportionate effects on the regulation of the homeostasis of the brain, making increased adhesion a risk factor (Cunnington et al., 2013; Moxon et al., 2009; Storm and Craig, 2014). In addition to these general effects, specific consequences for the brain may occur due to modulation of signaling mediated by ICAM-1 and EPCR, as EPCR-mediated signaling and ICAM-1 expression are linked by a complex interplay that might also contribute to cerebral disease. Infected erythrocytes prevent EPCR from interacting with its natural ligands, inhibiting signaling pathways that induce anti-inflammatory responses and protect the integrity of the endothelium (Petersen et al., 2015; Turner et al., 2013). Indeed, inflammation and brain swelling both occur in CM and are important in disease development. In addition, blockage of EPCR signaling increases expression of several PfEMP1 adhesion receptors, including ICAM-1 (Moxon et al., 2013). Paradoxically, parasite sequestration in the brain is also linked to loss of EPCR, removing an important regulator of pro-coagulation responses (Moxon et al., 2013). The capacity to bind to both receptors may give the parasite more potential to resist endothelial changes in response to binding (i.e., downregulation of EPCR) and to remain sequestered in the brain. Together with the increased expression of ICAM-1 on endothelial cells, this could lead to a vicious cycle that further exacerbates disease.

CM is a complex disease, and in addition to dual-binding PfEMP1, various other aspects of both host and parasite biology will influence the development of cerebral symptoms. Other surface protein families, including STEVORs, RIFINs, and the PfEMP1 involved in rosetting (the clustering of infected and uninfected erythrocytes), have been implicated in severe malaria (Doumbo et al., 2009; Goel et al., 2015; Niang et al., 2014; Rowe et al., 1997) and further study is required to assess whether they too have a significant impact on the development of cerebral symptoms. In addition, host genetic factors, such as erythrocyte surface protein polymorphisms, could affect the capacity of the parasite to invade erythrocytes, altering parasitemia and therefore severity (Band et al., 2015). Nevertheless, the identification of dual-binding PfEMP1 as a risk factor for CM highlights these molecules as a promising and tractable candidate for the development of a vaccine to contribute to the prevention of this form of the disease.

A major challenge in targeting PfEMP1s through vaccination is their high sequence variability, driven by a diversifying selection

pressure to evade immune detection. This is demonstrated by structural studies of CIDRα domains bound to EPCR or CD36 that revealed their interaction sites to retain their overall chemical nature, while significantly diversifying in sequence (Hsieh et al., 2016; Lau et al., 2015). This reduces the likelihood of the natural development of cross-inhibitory antibody responses that target these critical surfaces of CIDR domains. In contrast, the ICAM-1-binding site of the group A PfEMP1 is remarkably conserved, with a number of absolutely or predominantly conserved amino acid residues that make critical, direct interactions with ICAM-1 (Figure 2G). Indeed, we find that plasma from *P. falciparum*-exposed individuals contains IgG with the capacity to inhibit ICAM-1 binding by a broad range of motif-containing DBLβ domains (Figure 4). This encourages future efforts to raise broadly reactive antibody responses against these domains. This study therefore highlights stronger endothelial adhesion capacity to both ICAM-1 and EPCR as a risk factor for development of CM and identifies a group of PfEMP1s with a remarkably conserved ICAM-1-binding site as potential vaccine immunogen for use as part of a strategy to prevent death due to CM.

EXPERIMENTAL PROCEDURES

More detailed methods and references are provided in the Supplemental Experimental Procedures.

Crystal Structure Determination

The PF11_0521 DBLβ3_D4 domain was produced as described in the Supplemental Experimental Procedures and was mixed with a 1.5 molar excess of ICAM-1^{D1D2} before size exclusion chromatography. Crystals were grown using sitting-drop vapor diffusion with a well solution of 10% (w/v) PEG 20000, 20% (v/v) PEG 500, and 0.1 M Tris-BICINE (pH 8.5) and cryo-cooled in well solution with an increased PEG 500 concentration of 32%.

Data were collected on beamline I02 (Diamond Light Source), indexed and refined using iMosflm, and scaled using SCALA (Evans and Murshudov, 2013). Molecular replacement was performed using Phaser-MR (McCoy et al., 2007) with a trimmed DBLα domain (PDB: 2XU0) and individual D1 and D2 domains from ICAM-1^{D1D2} (PDB: 1IC1) as search models. This identified one copy of the complex in the asymmetric unit. The structure was built and refined through iterative cycles of refinement using Refmac5 (Murshudov et al., 2011), Buster (Bricogne et al., 2016), and model building in Coot (Emsley et al., 2010).

Surface Plasmon Resonance

Surface plasmon resonance experiments were carried out in a Biacore T200 instrument (GE Healthcare). ICAM-1^{D1D5}-Fc was bound to a CM5 chip (GE Healthcare) pre-coupled to protein A while EPCR was biotinylated by BirA and coupled to a biotin CAPture chip (GE Healthcare). Binding partners were injected for 240 s with a dissociation time of 660 s. To analyze dual binding to ICAM-1 and EPCR, ICAM-1^{D1D5}-Fc was immobilized. Two- and three-domain PfEMP1 constructs were then injected, followed by a concentration series of EPCR. Kinetic sensorgrams were fitted to a 1:1 interaction model to determine kinetic rate constants and dissociation constant.

Small-Angle X-Ray Scattering

Small-angle X-ray scattering data were collected on beamline BM29 at the European Synchrotron Radiation Facility and processed using the ATSAS processing suite. The resulting ab initio model was converted into an envelope before manual model docking.

Patient Samples

Plasmodium falciparum isolates were collected from malaria patients at hospitals in Ghana, Tanzania, and Benin. Clinical manifestations of malaria were classified according to the definitions and associated criteria of the World Health Organization. CM was identified by a positive blood smear of the asexual form of *P. falciparum* and unrousable coma (Blantyre coma score, BCS $\leq$ 2) with exclusion of other causes of coma and severe illness. SA was identified by hemoglobin < 5g/dL and BCS > 2. OM was indicated by BCS > 2 and hemoglobin > 5 g/dL. These studies were approved by the respective ethics committees. Additional plasma samples were collected from *P. falciparum*-exposed children during a cross-sectional village study in Tanzania and from *falciparum*-exposed adult Liberians (Lusingu et al., 2004; Theisen et al., 2000).

Antibody Production and Purification

Rat antibodies were obtained by immunization with DBLβ domains, followed by affinity purification of IgG. Human antibodies against DBLβ domains or the ICAM-1-binding motif were affinity purified from pooled plasma from nine Liberian adults.

ELISA

ICAM-1 binding was assessed by ELISA using DBLβ domain-coated Maxisorp plates, with detection using ICAM-1^{D1D5}-Fc and rabbit anti-human IgG-HRP (Dako). A similar assay was used to assess the reactivity of human plasma samples to the DBLβ domains or to assess their efficacy at blocking ICAM-1 binding.

Assessment of Infected Erythrocyte Adhesion to Receptors

Plasmodium falciparum parasites were maintained in in vitro blood culture and selected for specific PfEMP1 expression using antibodies. Microslides (Ibidi) were coated with recombinant receptors (ICAM-1^{D1D5}-Fc, EPCR, or CD36) or seeded with HBMEC (Sciencell). Infected erythrocytes were exposed to the microslides at a range of physiological shear stresses and bound infected erythrocytes were quantified by microscopy.

Immunofluorescence

Infected erythrocytes were incubated with complexes of ICAM-1^{D1D5}-Fc:anti-human IgG-Dylight 488 (Abcam) and/or EPCR:anti-his IgG (QIAGEN):anti-mouse IgG Alexa 568 (Abcam) before washing and analysis in a Zeiss AxioImager Z1.

Real-Time qPCR

Parasite RNA from clinical samples was reverse transcribed into DNA and subjected to real-time qPCR using primer pairs (Table S7C) designed to amplify ICAM-1 motif-containing *var* genes or *var* gene subclasses encoding EPCR-binding CIDRα1 domains.

Sequence Analysis

DBLβ domain sequences (Rask et al., 2010) were used to blast extract DBLβ sequences from assemblies of Illumina whole-genome sequencing data, including MalariaGEN data (Miotto et al., 2015) and assembled amplicons from eight Ghanaian isolates. Those predicted to bind ICAM-1 were identified using search term ASNGGPGYYNTEVQK. A database containing sequences of the six most expressed *var* genes in 45 Tanzanian children with severe or uncomplicated malaria (Jespersen et al., 2016) was used to correlate relative expression of motif-containing *var* genes when compared with disease outcome. *Var* gene expression and disease severity were analyzed using the Wilcoxon-Mann-Whitney rank-sum test. To determine whether study site affected the outcome, a logistical regression model was used.

ACCESSION NUMBERS

Sequences originating from the present study are deposited in GenBank with accession numbers GenBank: KF984156, KJ866957, KJ866958, KJ866959, KM364031, KM364032, KM364033, and KM364034. Structure files have been deposited in the PDB under ID code PDB: 5MZA.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, seven figures, and seven tables and can be found with this article online at http://dx.doi.org/10.1016/j.chom.2017.02.009.

AUTHOR CONTRIBUTIONS

F.L., Y.A., A.B., T.L., M.K.H., and A.T.R.J. conceived and planned the study. F.L., Y.A., L.H., M.K.H., and A.T.R.J. wrote the manuscript. F.L., A.B., R.W.O., L.T., B.G., S.N.-S., and A.T.R.J. contributed with recombinant proteins and antibodies. F.L. purified and crystallized proteins and collected and analyzed small-angle X-ray scattering data. F.L. and M.K.H. prepared crystals, collected data, and solved the structure. F.L. and C.K.Y.L. performed and analyzed surface plasmon resonance experiments. Y.A. performed adhesion assays and IFA. A.B. and J.E.V.P. did parasite culturing and flow cytometry analysis. R.W.O. and G. E.-M. performed ELISA studies. R.W.O. and A.T.R.J. did real-time qPCR work. J.S.J., T.L., and A.T.R.J. did the sequencing and bioinformatics analysis. N.T.N., G.E.-M., A.M., M.F.O., J.P.L., C.W.W., and T.G.T. organized clinical work and processed clinical samples. All authors read and commented on the manuscript. F.L., Y.A., A.B., M.K.H., and A.T.R.J. contributed equally to the work.

ACKNOWLEDGMENTS

M.K.H. is a Wellcome Trust Investigator. A.B., A.T.R.J., C.W.W., G.E.-M., J.E.V.P., J.S.J., L.H., M.F.O., R.W.O., T.L., and Y.A. were supported by Novo Nordisk Fonden (NNF13OC0006249), Hørslev-Fonden, Augustinus Fonden (12-0378 and 15-3000), Lundbeckfonden (R180-2014-3098 and R140-2013-13448), Aase and Ejnar Danielsens Fond, Oda og Hans Svenningsens Fond (BOF-10109), Axel Muusfeldts Fond, Grosserer L.F. Foghts Fond, University of Copenhagen UCPH2016 Programme of Excellence, the Consultative Committee for Development Research (DANIDA), the Danish Council for Independent Research (4004-00032, T1333-00220, 1331-00089B, and Sapere Aude program DFF–4004-00624B), and the NIH (R01HL130678). Fieldwork in Benin was supported by PEERS-EPIVAC grant from AIRD/IRD. We thank the Beninese, Ghanaian, and Tanzanian donors; the MalariaGen community project for the use of data (Miotto et al., 2015); Michael Theisen for immune plasma from Liberia; and Mette Ulla Madsen and Maiken Høwning Visti for excellent technical assistance. We acknowledge the Core Facility for Integrated Microscopy, Faculty of Health and Medical Sciences, University of Copenhagen for use of the Zeiss AxioImage Z1 and Zachary Barbati for doing initial experiments not included in this paper. We thank David Staunton for assistance with biophysical experiments and Ed Lowe and the beamline scientists at I02 at Diamond Light Source for assistance with data collection. The accession codes for the sequences originating from Jespersen et al. (2016) are GenBank: KX154876, KX154960, KX154911, KX154945, KX154859, KX154816, KX154936, KX154871, KX154901, KX154818, KX154872, KX154935, and KX154836.

Received: October 4, 2016
Revised: January 20, 2017
Accepted: February 10, 2017
Published: March 8, 2017

REFERENCES

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., and 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. USA *109*, E1782–E1790.

Avril, M., Bernabeu, M., Benjamin, M., Brazier, A.J., and Smith, J.D. (2016). Interaction between endothelial protein C receptor and intercellular adhesion molecule 1 to mediate binding of Plasmodium falciparum-infected erythrocytes to endothelial cells. MBio *7*, e00615-16.

Band, G., Rockett, K.A., Spencer, C.C., and Kwiatkowski, D.P.; Malaria Genomic Epidemiology Network (2015). A novel locus of resistance to severe malaria in a region of ancient balancing selection. Nature *526*, 253–257.

Bengtsson, A., Joergensen, L., Rask, T.S., Olsen, R.W., Andersen, M.A., Turner, L., Theander, T.G., Hviid, L., Higgins, M.K., Craig, A., et al. (2013). A novel domain cassette identifies Plasmodium falciparum PfEMP1 proteins binding ICAM-1 and is a target of cross-reactive, adhesion-inhibitory antibodies. J. Immunol. *190*, 240–249.

Bernabeu, M., Danziger, S.A., Avril, M., Vaz, M., Babar, P.H., Brazier, A.J., Herricks, T., Maki, J.N., Pereira, L., Mascarenhas, A., et al. (2016). Severe adult malaria is associated with specific PfEMP1 adhesion types and high parasite biomass. Proc. Natl. Acad. Sci. USA *113*, E3270–E3279.

Birbeck, G.L., Molyneux, M.E., Kaplan, P.W., Seydel, K.B., Chimalizeni, Y.F., Kawaza, K., and Taylor, T.E. (2010). Blantyre Malaria Project Epilepsy Study (BMPES) of neurological outcomes in retinopathy-positive paediatric cerebral malaria survivors: a prospective cohort study. Lancet Neurol. *9*, 1173–1181.

Bricogne, G., Blanc, E., Brandl, M., Flensburg, C., Keller, P., Paciorek, W., Roversi, P., Sharff, A., Smart, O.S., Vonrhein, C., and Womack, T.O. (2016). BUSTER version 2.10.2 (Global Phasing).

Claessens, A., Adams, Y., Ghumra, A., Lindergard, G., Buchan, C.C., Andisi, C., Bull, P.C., Mok, S., Gupta, A.P., Wang, C.W., et al. (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. USA *109*, E1772–E1781.

Cunnington, A.J., Riley, E.M., and Walther, M. (2013). Stuck in a rut? Reconsidering the role of parasite sequestration in severe malaria syndromes. Trends Parasitol. *29*, 585–592.

Dondorp, A.M., Fanello, C.I., Hendriksen, I.C., Gomes, E., Seni, A., Chhaganlal, K.D., Bojang, K., Olaosebikan, R., Anunobi, N., Maitland, K., et al.; AQUAMAT group (2010). Artesunate versus quinine in the treatment of severe falciparum malaria in African children (AQUAMAT): an open-label, randomised trial. Lancet *376*, 1647–1657.

Doumbo, O.K., Thera, M.A., Koné, A.K., Raza, A., Tempest, L.J., Lyke, K.E., Plowe, C.V., and 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*, 987–993.

Emsley, P., Lohkamp, B., Scott, W.G., and Cowtan, K. (2010). Features and development of Coot. Acta Crystallogr. D Biol. Crystallogr. *66*, 486–501.

Evans, P.R., and Murshudov, G.N. (2013). How good are my data and what is the resolution? Acta Crystallogr. D Biol. Crystallogr. *69*, 1204–1214.

Goel, S., Palmkvist, M., Moll, K., Joannin, N., Lara, P., Akhouri, R.R., Moradi, N., Öjemalm, K., Westman, M., Angeletti, D., et al. (2015). RIFINs are adhesins implicated in severe Plasmodium falciparum malaria. Nat. Med. *21*, 314–317.

Howell, D.P., Levin, E.A., Springer, A.L., Kraemer, S.M., Phippard, D.J., Schief, W.R., and Smith, J.D. (2008). Mapping a common interaction site used by *Plasmodium falciparum* Duffy binding-like domains to bind diverse host receptors. Mol. Microbiol. *67*, 78–87.

Hsieh, F.L., Turner, L., Bolla, J.R., Robinson, C.V., Lavstsen, T., and Higgins, M.K. (2016). The structural basis for CD36 binding by the malaria parasite. Nat. Commun. *7*, 12837.

Hviid, L., and Jensen, A.T. (2015). PfEMP1—a parasite protein family of key importance in *Plasmodium falciparum* malaria immunity and pathogenesis. Adv. Parasitol. *88*, 51–84.

Idro, R., Jenkins, N.E., and Newton, C.R. (2005). Pathogenesis, clinical features, and neurological outcome of cerebral malaria. Lancet Neurol. *4*, 827–840.

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

Jensen, A.T., Magistrado, P., Sharp, S., Joergensen, L., Lavstsen, T., Chiucchiuini, A., Salanti, A., Vestergaard, L.S., Lusingu, J.P., Hermsen, R., et al. (2004). *Plasmodium falciparum* associated with severe childhood malaria preferentially expresses PfEMP1 encoded by group A *var* genes. J. Exp. Med. *199*, 1179–1190.

Jespersen, J.S., Wang, C.W., Mkumbaye, S.I., Minja, D.T., Petersen, B., Turner, L., Petersen, J.E., Lusingu, J.P., Theander, T.G., and Lavstsen, T. (2016). Plasmodium falciparum var genes expressed in children with severe malaria encode CIDRα1 domains. EMBO Mol. Med. *8*, 839–850.

Kim, J.H., Singvall, J., Schwarz-Linek, U., Johnson, B.J., Potts, J.R., and Höök, M. (2004). BBK32, a fibronectin binding MSCRAMM from Borrelia burgdorferi, contains a disordered region that undergoes a conformational change on ligand binding. J. Biol. Chem. *279*, 41706–41714.

IAI Accepted Manuscript Posted Online 5 February 2018
Infect. Immun. doi:10.1128/IAI.00622-17

Natural and vaccine-induced acquisition of cross-reactive IgG inhibiting ICAM-1-specific binding of a PfEMP1 subtype associated specifically with cerebral malaria

Rebecca W. Olsen,[a*] Gertrude Ecklu-Mensah,[a,d*] Anja Bengtsson,[a] Michael F Ofori,[d] John P.A. Lusingu, [e] Filip C. Castberg,[a,b] Lars Hviid,[a,c] Yvonne Adams,[a] and Anja T.R. Jensen [a#]

Centre for Medical Parasitology at Department of Immunology and Microbiology, Faculty of Health and Medical Sciences, University of Copenhagen [a] and at Department of Clinical Microbiology [b] and Department of Infectious Diseases [c], Copenhagen University Hospital (Rigshospitalet), Denmark; Department of Immunology, Noguchi Memorial Institute for Medical Research, University of Ghana, Legon, Ghana [d]; National Institute for Medical Research, Tanga Centre, Tanzania [e]

*shared first-authorship.

#Address correspondence to: Anja TR Jensen, atrj@sund.ku.dk

Running title: Cross-reactive IgG inhibits CM-type IE adhesion to ICAM-1

Word count abstract: 250

Word count text: 4,921

ABSTRACT

Cerebral malaria (CM) is a potentially deadly outcome of *Plasmodium falciparum* malaria that is precipitated by sequestration of infected erythrocytes (IEs) in the brain. The adhesion of IEs to brain endothelial cells is mediated by a subtype of parasite-encoded PfEMP1 that facilitate dual binding to host ICAM-1 and EPCR. The PfEMP1 subtype is characterized by the presence of a particular motif (DBLβ_motif) in the constituent ICAM-1-binding DBLβ domain. The rate of natural acquisition of DBLβ_motif-specific IgG and the ability to induce such antibodies by vaccination are unknown, and the aim of this study was to provide such data.

We used ELISA to measure DBLβ-specific IgG in plasma from Ghanaian children with malaria. The ability of human immune plasma and DBLβ-specific rat anti-sera to inhibit the interaction between ICAM-1 and DBLβ was assessed using ELISA and *in vitro* assays of IE adhesion under flow.

Acquisition of DBLβ_motif-specific IgG coincided with age-specific susceptibility to CM. Broadly cross-reactive antibodies inhibiting the interaction between ICAM-1 and DBLβ_motif domains were detectable in immune plasma and in sera of rats immunized with specific DBLβ_motif antigens. Importantly, antibodies against the DBLβ_motif inhibited ICAM-1-specific *in vitro* adhesion of erythrocytes infected by four of five *P. falciparum* isolates from cerebral malaria patients. We conclude that natural exposure to *P. falciparum* as well as immunization with specific DBLβ_motif antigens can induce cross-reactive antibodies that inhibit the interaction between ICAM-1 and a broad range of DBLβ_motif domains. These findings raise hope that a vaccine designed specifically to prevent CM is feasible.

Keywords. *Plasmodium falciparum;* PfEMP1; DBLβ cross-reactive antibodies; ICAM-1 binding motif; adhesion inhibition.

INTRODUCTION

Plasmodium falciparum is the major cause of the estimated 430,000 deaths due to malaria reported annually (1). The pathogenesis of *P. falciparum* is linked to sequestration of IEs in various tissues, which can lead to tissue-specific inflammation, circulatory obstruction, and organ dysfunction (reviewed in ref. 2). IE sequestration is mediated by members of the erythrocyte membrane protein 1 (PfEMP1) family. These proteins are encoded by approximately 60 *var* genes per *P. falciparum* genome, and are expressed on the IE surface where they bind to a range of host receptors (reviewed in ref. 3).

Despite extensive inter- and intra-clonal diversity, the PfEMP1 proteins can be classified into three major groups (A, B and C), based on *var* gene sequence and chromosomal context (4, 5). Group A is less diverse than the other groups, and expression of Group A PfEMP1 proteins on the IE surface has repeatedly been linked to the development of severe malaria (6, 7). This is consistent with the restricted serological diversity of *P. falciparum* parasites from patients with severe malaria (8, 9). It also fits the observation that acquisition of immunity to complicated disease often precedes development of protection from uncomplicated malaria and asymptomatic parasitemia, and that PfEMP1 expression is modulated by PfEMP1-specific immunity (10-12). More recently, the PfEMP1 Groups have been further sub-divided according to their constituent Duffy-binding-like (DBL) and cysteine-rich inter-domain region (CIDR) domains, and a number of multi-domain blocks, known as domain cassettes (DCs), have been identified (13-16). Three of these, (DC4, DC8, and DC13) have been linked to severe malaria in children (6, 14, 17, 18). DC4 consists of three domains (DBL$\alpha_{1.1/1.4}$-CIDR$\alpha_{1.6}$-DBLβ_3) and defines a subfamily of Group A PfEMP1 proteins that mediates binding to intercellular adhesion molecule 1 (ICAM-1) (15). IE adhesion to ICAM-1 appears associated with severe malaria, implicating DC4-specific antibodies in clinical protection as they are acquired early in life by children living in malaria endemic areas and are associated with

clinical protection from malaria (6, 15, 19). However, until recently the role of IE adhesion to ICAM-1 specifically in CM was unclear (20-24).

DC8 consists of four domains (DBLα_2-CIDR$\alpha_{1.1}$-DBLβ_{12}-DBL$\gamma_{4/6}$) and is found among group B/A genes, while the two-domain (DBL$\alpha_{1.7}$-CIDR$\alpha_{1.4}$) DC13 is found in some group A PfEMP1 proteins (14). Endothelial protein receptor C (EPCR) is the cognate receptor for DC8- and DC13-containing PfEMP1 (25). Some studies have reported high transcript levels of *var* genes encoding EPCR-binding PfEMP1 variants in parasites from children with severe malaria, including CM, and perturbed EPCR expression in brain tissue of CM patients (26-28). While these findings point to a role for EPCR in severe malaria in general, and CM in particular, available evidence overall remains equivocal (29-31).

We have previously proposed that the above ambiguities may be reflecting that the pathogenesis of CM involves *P. falciparum* parasites expressing PfEMP1 capable of mediating IE adhesion to both ICAM-1 (via DBLβ) and EPCR (via CIDRα_1) (3). A few such dual receptor-binding PfEMP1 proteins were identified shortly after, although the study did not link them to CM specifically, and did not document concomitant binding to both receptors (32). However, those gaps were recently closed by our demonstration of a link between CM and Group A PfEMP1 proteins capable of binding ICAM-1 and EPCR simultaneously (33). This dual receptor-binding sub-group of PfEMP1 proteins is characterized by an EPCR-binding CIDRα_1 domain followed immediately by a DBLβ domain featuring a specific ICAM-1-binding motif (DBLβ_motif domain) (33).

The rate of natural acquisition of IgG against the DBLβ_motif associated specifically with CM, and the ability to induce such antibodies by vaccination, are both unknown. The current study was therefore designed to investigate if cross-reactive IgG specific for DBLβ_motif domains and/or their ICAM-1-binding motif are acquired following natural exposure to *P. falciparum* parasites, and if ICAM-1 adhesion-inhibitory antibodies can be induced by immunization with specific

DBLβ_motif proteins and with peptides representing the ICAM-1-binding motif therein. Confirmation of these hypotheses would support the feasibility of developing a vaccine designed specifically to prevent CM.

MATERIALS AND METHODS

Plasma and parasite samples

Plasma samples (n= 79) for the present study were collected in 2014 at Hohoe Municipality Hospital in the Volta Region of Ghana from children with acute malaria (Table 1) (33, 34). *P. falciparum* parasites were collected at this hospital (n= 14) as well as at the Korogwe District Hospital (n= 19) in Korogwe District in Northeastern Tanzania (35). Clinical manifestations of malaria were classified according to the definitions and associated criteria of the World Health Organization. Patients were categorized as having cerebral malaria (CM; n= 7) if they had a positive blood smear of the asexual form of *P. falciparum*, unrousable coma (Blantyre coma score, BCS ≤2) with exclusion of other causes of coma and severe illness. Patients were categorized as having severe malarial anemia (SA; n=12) if haemoglobin <5 g/dL and BCS >2. Patients were classified as having severe malaria other than SA and CM if they presented with hyperparasitaemia (>250,000 parasites/µL), multiple convulsions (>2 episodes in 24 h), respiratory distress (i.e., rapid, deep, and labored breathing) or combinations of these symptoms. Patients with uncomplicated malaria (UM; n= 48) had less than 250,000 parasites/µL.

The study was approved by the Ethical Review Committee of the Ghana Health Services (file GHS-ERC 08/05/14) and by the National Ethical Review Committee of the National Institute for Medical Research, Tanzania (NIMR/HQ/R.8a/Vol.IX/559). A pool of plasma from *P. falciparum*-exposed Tanzanian individuals (36) and 25 Danish non-exposed individuals were used as positive and negative controls, respectively. Long-term *in vitro* culture-adapted and fully sequenced parasite clones 3D7, HB3, and IT4 were also studied.

Recombinant proteins

The genes encoding the DBLβ domains used were amplified from genomic DNA or produced as synthetic genes (http://eurofins.dk) (Table 3). Amplicons were sub-cloned into a modified pET15b vector and expressed as his-tagged proteins in *E. coli* Shuffle C3030 cells (https://neb.com) as described (15). All the proteins were purified (Fig. S1) by immobilized metal ion affinity chromatography using HisTrap HP 1 mL columns (GE Healthcare), and are referred to by the codes listed in Table 3.

Recombinant Fc-tagged ICAM-1 was expressed in HEK293 cells and purified on a HiTrap Protein G HP (http://www3.gehealthcare.dk/) as described (37).

DBLβ-specific anti-sera

We generated rat antisera to recombinant proteins M1, M6, M9, M10, N27 and N33 (Table 3), and to two synthetic peptides (https://schafer-n.com/) that corresponded to the ICAM-1-binding motifs in M6 (M6pep: LYAKARIVASNGGPGYYNTEVQKKDRSVYDFLYELHLQNGGKKGPPPATHPYKSVNTRD KRDATDDTTP) and M9 (M9pep: LYKEAEIYARNGGPGYYNTEVQKEDKPVVDFLYELHLQNGGKKGPP AATHPSKSVTTRVKRDTTVDTPS). M1, M6, M9 and M10 were selected from different branches of the previously published phylogenic tree of DBLβ domains, to represent dual ICAM-1- and EPCR-binding Group A PfEMP1 proteins (33). In a similar way, N27 and N33 were chosen as random examples of ICAM-1-binding group B PfEMP1 proteins.

In each case, Wistar rats were immunized with the antigen (25 µg) in Freund's incomplete adjuvant followed by two booster vaccinations two weeks apart (15 µg/boost). Blood was collected two weeks after the last immunization. All animal procedures were approved by The Danish Animal Procedures Committee ("Dyreforsøgstilsynet") as described in permit no. 2013-15-2934-

00920, and all experiments were done according to the guidelines described in Danish act LBK 1306 (23/11/2007) and BEK 1273 (12/12/2005).

IgG purification

IgG specific for M6 and M6pep were affinity-purified from rat antisera as described (38). In brief, M6 and M6pep (1 mg/mL) were dialysed overnight against coupling buffer and coupled to Hi-trap NHS-activated HP columns, as described by the manufacturer (http://www3.gehealthcare.dk/). Anti-sera were diluted 1:1 in PBS and affinity-purified on the columns, followed by elution of bound IgG in low-pH buffer (glycine/HCl, pH 2.75) and pH adjustment by Tris/HCl (1 M, pH 9.0).

Measurements of DBLβ-specific IgG levels

MaxiSorp microtiter plates (http://www.sigmaaldrich.com/) were coated with recombinant DBLβ domains (50 µL; 5 µg/mL) as described previously (15). Plasma samples (diluted 1:100 in blocking buffer) were incubated (50 µL/well, 1 h, room temperature) in duplicate wells. The plates were washed (PBS + 1% Triton X-100), and bound antibody was detected with HRP-conjugated anti-human IgG (1:3,000 in blocking buffer) (http://www.agilent.com). After incubation (1 h) and washing as above, bound detection antibody was detected using OPD tablets, according to the manufacturer's instructions (http://www.agilent.com). The OD values were read at 490 nm using a VERSAmax microplate reader (http://www.moleculardevices.com/). Antibody reactivity was expressed in arbitrary ELISA units (EU) calculated as $(OD_{sample} - OD_{background})/(OD_{positive\ control} - OD_{background}) \times 100$ (ref. 39).

Measurements of antibody-mediated inhibition of DBLβ binding to ICAM-1

Inhibition of recombinant DBLβ domain binding to ICAM-1 by human immune plasma and rat anti-sera was measured by ELISA. In brief, wells of MaxiSorp plates were coated with

recombinant ICAM-1 (ref. 37) (50 µL/well; 2 or 4 µg/mL; 0.1M glycin/HCl buffer; pH 2.75) by incubation overnight (4°C) and blocked with blocking buffer (1 h; room temperature). His-tagged DBLβ proteins (0.5-16 µg/mL final concentration) were mixed with immune plasma or anti-sera (1:5 final concentration) or purified IgG (10 µg/mL final concentration) and added to duplicate wells (1 h; room temperature). The plates were washed and binding detected using HRP-conjugated anti-penta-his antibody (http://www.qiagen.com/) as described above. All antisera were prescreened by ELISA to verify absence of his-tag-reactive antibodies.

In vitro *culture and antibody selection of* P. falciparum *parasites*

The *P. falciparum* clones 3D7, HB3, and IT4 were maintained in long-term *in vitro* cultures, and antibody-selected for IE surface expression of specific PfEMP1 proteins as described (15, 38). In brief, we used the human monoclonal IgG antibody AB01 to select 3D7 IEs for expression of PFD1235w (40). HB3 IEs were similarly selected for surface expression of VAR03 using a rat anti-serum against M8, and IT4 IEs for expression of VAR13 by a rat anti-serum against N27 (ref. 33). In all cases, expression of the required PfEMP1 on the surface of MACS-purified mature IEs was monitored by flow cytometry using PfEMP1-specific antisera, essentially as described (38, 41). Only cultures with >60% antibody-labeled IEs were used.

In addition, primary isolates of *P. falciparum* parasites from 33 of the above-mentioned malaria patients were cultured *in vitro* for up to 28 days (median; 25 and 75% percentile [8 days; 2.5 and 13 days]) in Albumax (10%) (http://www.thermofisher.com/), supplemented with normal human serum (NHS, 2%), essentially as described (42). The genotypic identity of the isolates was routinely verified by genotyping as described (43), and *Mycoplasma* infection was regularly excluded using the MycoAlert *Mycoplasma* Detection Kit (http://www.lonza.com/) according to the manufacturer's instructions.

***Adhesion of IEs to ICAM-1 under physiological flow* in vitro**

Microslides ($VI^{0.1}$) (http://www.ibidi.com/) were coated with recombinant ICAM-1-Fc protein (37) (50 µg/mL, 4°C, overnight) and blocked using PBS plus 2% BSA. Parasite suspensions, adjusted to 3% parasitemia and 1% hematocrit in RPMI-1640 supplemented with 2% NHS (pH 7.2), were flowed over the coated slides (5 min) at a shear stress of 1 dyn/cm^2 as described (44). Bound IE/mm^2 in five separate fields were counted, using a Leica inverted phase contrast microscope (20× magnification). To assess the capacity of affinity-purified DBLβ-specific IgG to inhibit adhesion, IEs selected for expression of particular PfEMP1 variants were pre-incubated with the purified IgG (15 min, room temperature). The receptor specificity of the IE adhesion observed was verified by pre-incubating the ICAM-1-coated flow channels with an ICAM-1-specific antibody (40 µg/mL, clone 15.2, AbD Serotec).

Inhibition of ICAM-1-adhering (≥ 10 adherent IEs/mm^2) erythrocytes infected with primary *P. falciparum* field isolates was tested using pooled rat antisera (1:100 dilution) to two peptides representing the ICAM-1-binding motifs in M6 (M6pep) and M9 (M9pep). A minimum of three independent experiments were completed for each of the tested laboratory clones (3D7, HB3, IT4), whereas each of the field isolates was tested in one experiment with five technical replicates. All assays were blinded to the operator.

Immunofluorescence microscopy of IEs labeled with PfEMP1-specific antibodies

Immunofluorescence microscopy was done essentially as described (45). Briefly, aliquots (50 µL) of erythrocytes infected by parasites expressing PFD1235w, HB3VAR03, or IT4VAR13, respectively were adjusted to 5% parasitemia and resuspended in PBS containing 1% Ig-free BSA (https://www.sigmaaldrich.com). Antisera were added (1:50 dilution) and incubated on ice (1 h). Following three washes, cells were resuspended and labeled with anti-rat-FITC secondary antibody (1:500) and incubated as before. Cells were washed three times and thin smears made. Nuclei were

visualized by adding 5 µL ProLong Gold anti-fade (http://www.thermofisher.com/) prior to addition of coverslips. Immunofluorescence was visualized with a Nikon Eclipse TE2000 microscope equipped with an ×63 objective.

Bioinformatics

Multiple alignments of DBLβ domains known to bind ICAM-1 were made using MUSCLE v. 3.7 software (46), and sequence distance trees made with MEGA software (47). A WebLogo 3 sequence logo (48) of the ICAM-1 binding motif was generated based on alignment of the included DBLβ_motif domains (Table 3) with the consensus motif: I[V/L]x3N[E]GG[P/A]xYx27GPPx3H (15, 33).

Statistics

We used Pearson product moment correlation (r) or Spearman rank-order correlation (r_s) to evaluate parameter association, and one-way analysis of variance (F), Kruskal-Wallis one-way analysis of variance on ranks (T), and Mann-Witney test (U) to test for inter-group differences.

RESULTS

Delayed acquisition of IgG to ICAM-1-binding Group A-type DBLβ domains

Group A PfEMP1 proteins that contain a DBLβ domain with the motif (I[V/L]x3N[E]GG[P/A]xYx27GPPx3H; DBLβ_motif domains) can bind to the host endothelial receptor ICAM-1 and always feature a neighboring CIDRα1 domain that enables concomitant binding to another endothelial receptor, EPCR (33). Expression of these dual receptor-binding PfEMP1 is associated with CM, which is a major cause of mortality and severe morbidity among African children. The age at which most CM cases occurs varies with transmission intensity, but generally falls later than the peak prevalence of parasitemia and malaria-related severe anemia. DBLβ domains present in Group A PfEMP1 proteins, but without the above motif (DBLβ_non-motif domains), do not bind ICAM-1 and are less conserved in the C-terminus (33).

We first used ELISA to measure levels of IgG with specificity for 14 recombinant DBLβ_motif domains (M1-M12, M14-M15; Fig. 1) and 13 non-motif DBLβ domains (N20-N32) in plasma from 79 Ghanaian children with different clinical presentations of *P. falciparum* malaria (Table 1). The antibody reactivity to all these Group A PfEMP1 proteins varied substantially among the children (Fig. 2A) and also among the different DBLβ domains (Fig. 2B). Overall, the plasma levels of IgG increased with age ($P(r)<0.001$), with levels of IgG specific for DBLβ_motif proteins being generally lower than DBLβ_non-motif-specific IgG ($P(T)<0.001$). However, this latter difference was mainly due to low IgG recognition of DBLβ_motif among the younger age groups (≤4 years-of-age) (Fig. 2C). Thus, levels of IgG specific for DBLβ_motif were significantly lower than DBLβ_non-motif-specific IgG levels among children aged 1-2 years and 3-4 years ($P(T)\leq0.001$), but not in the two older age classes considered (5-6 years and >6 years; $P(T)\geq0.21$).

Overall plasma levels of IgG specific for DBLβ_motif as well as DBLβ_non-motif domains were similar in patients with severe and uncomplicated malaria (P(U)=0.4 in both cases), while levels of DBLβ_non-motif domain-specific IgG were lower in the children with severe malaria than in the patients with uncomplicated disease (scores 7 and 12.5; P(U)=0. 02, respectively).

Taken together, these results indicate that DBLβ_motif and non-motif proteins are similarly immunogenic, but that acquisition of DBLβ_motif-specific IgG is acquired later in life than DBLβ_non-motif-specific IgG.

Immunization with DBLβ_motif antigens induces cross-reactive, neutralizing IgG

Plasma from clinically immune individuals living in areas of stable and intense transmission of *P. falciparum* parasites can inhibit the interaction between ICAM-1 and a range of DBLβ_motif domains (15, 33). In this study, a pool of plasma from 10 of the children (selected for reactivity with DBLβ_motif domains (Fig. 2A and Table 1) and plasma availability) inhibited ICAM-1 binding to DBLβ_motif protein M9 (Fig. 3A). These data suggest the presence of neutralizing IgG capable of recognizing multiple DBLβ_motif-containing PfEMP1 variants (cross-reactive IgG). If such antibodies could be induced by vaccination, it would increase the feasibility of developing a broadly protective PfEMP1-based vaccine against cerebral malaria. However, our data could also reflect the presence of many different variant-specific IgG specificities, where each antibody specificity is capable of inhibiting the binding of ICAM-1 to only a particular DBLβ_motif domain variant (a broad repertoire of IgG with narrow specificity). It is inherently difficult to distinguish between these two alternatives in naturally acquired immunity. Nevertheless, truly cross-reactive PfEMP1-specific human antibodies have previously been demonstrated in a study employing naturally acquired monoclonal IgG specific for the VAR2CSA-type PfEMP1 involved in the pathogenesis of placental malaria (49).

To investigate the relative importance of the above non-exclusive alternatives, to further assess the functional significance of DBLβ_motif in acquired immunity, and as a first step towards PfEMP1-based vaccination specifically against cerebral malaria, we immunized four rats with DBLβ_motif proteins M1, M6, M9, and M10, respectively (Table 3 and Fig. 1S). We used ELISA to test the ability of the anti-sera to inhibit binding of ICAM-1 to 14 DBLβ_motif proteins (M1-M7 and M9-M15) and two ICAM-1-binding DBLβ_non-motif proteins (N27 and N33). Each of the four DBLβ_motif-specific anti-sera inhibited binding of ICAM-1 to most of the DBLβ_motif proteins by more than 50%, but had little effect on ICAM-1-binding to DBLβ_non-motif domains (Fig. 3B). When pooled, the DBLβ_motif-specific anti-sera strongly inhibited (>75%) binding of ICAM-1 to all DBLβ_motif domains, with much less effect (<50%) on ICAM-1-binding to the DBLβ_non-motif domains (Fig. 3B). We next affinity-purified IgG from three of the rat anti-sera, using M6pep, to evaluate the involvement of IgG directly targeting the ICAM-1 binding region in the above inhibition. The purified M6pep-specific IgG generally inhibited ICAM-1 binding to the same degree as the anti-sera (Fig. 3B), with strong correlation between the anti-serum and motif-specific IgG data for M6 (r_s=0.78; P<0.001).

Overall, these data indicate that immunization with single DBLβ_motif antigens can induce cross-reactive IgG that inhibits binding of ICAM-1 to the homologous as well as a broad range of heterologous DBLβ_motif domains.

DBLβ_motif-specific IgG is broadly inhibitory of IE adhesion to ICAM-1 under physiologic flow

The ability of neutralizing IgG to interfere with receptor-specific IE sequestration *in vivo* likely depends on the characteristics of the involved PfEMP1 *per se*, on their expression on the IE surface, as well as on the shear forces at the anatomical location of the interaction of IEs with host endothelium. We have previously shown that naturally acquired IgG can inhibit ICAM-1-specific

adhesion of erythrocytes infected by *P. falciparum* expressing DBLβ_motif-containing PfEMP1 (33). To test if such antibodies could also be elicited by immunization with recombinant PfEMP1 proteins containing DBLβ_motif, we tested the ability of DBLβ_motif-specific IgG to inhibit adhesion of IEs to ICAM-1 in an *in vitro* assay simulating physiologic flow conditions (44). M6-specific IgG significantly inhibited ICAM-1-specific adhesion of IEs expressing the homologous PfEMP1 (PFD1235w; P(F)<0.001; Fig. 4A) or a heterologous PfEMP1 (HB3VAR03; P(F)<0.001; Fig. 4B). This was also the case for IgG specific for M9 and M10 (Table 3) and IgG purified on the ICAM-1-binding motif in M6 (M6pep; P(F)<0.001). The M9-specific antibodies also affected ICAM-1-specific adhesion of IEs expressing IT4VAR13, a Group B PfEMP1 protein that binds ICAM-1 but does not contain DBLβ_motif (ref. 33) (Fig. 4C). Conversely, an anti-serum to the ICAM-1-binding domain in IT4VAR13 (N27) inhibited ICAM-1-specific adhesion of the homologous IEs (Fig. 4C), but had no effect on ICAM-1-specific adhesion of IEs expressing the DBLβ_motif-containing PfEMP1 proteins PFD1235w (Fig. 4A) or HB3VAR03 (Fig. 4B).

We conclude from these experiments that immunization with DBLβ_motif antigens induce cross-reactive IgG that inhibit ICAM-1-specific adhesion of IEs that express a variety of PfEMP1 proteins containing DBLβ_motif domains. The inhibition of native PfEMP1 protein to ICAM-1 under conditions of flow thus mirrors that observed with recombinant proteins in ELISA.

Immunization with peptides representing the ICAM-1-binding region induces antibodies broadly inhibiting the binding of recombinant and native DBLβ_motif domains to ICAM-1

The results above suggested that IgG targeting the ICAM-1-binding region in DBLβ_motif domains is of particular importance for inhibiting the binding of Group A dual receptor-binding PfEMP1 to ICAM-1. This interpretation is further supported by our recent data showing that DBLβ_motif-purified antibodies from naturally infected humans and experimentally vaccinated

animals inhibit ICAM-1-specific adhesion of IEs expressing the DBLβ_motif-containing PfEMP1 protein PFD1235w (33). To assess directly if inhibitory and cross-reactive antibodies could be elicited by peptide immunization, we immunized rats with peptides representing the ICAM-1-binding region in DBLβ_motif domains (M6pep and M9pep) and tested their ability to inhibit binding of ICAM-1 to DBLβ_motif domains. Antisera from rats immunized with M6pep only, or with M6pep and M9pep, were broadly inhibitory of the binding of ICAM-1 to 10 DBLβ_motif domains (M2-M7, M9, M11-M13). The peptide antisera did not affect binding to two ICAM-1-binding DBLβ_non-motif domains (N27 and N33) (Fig. 5A). Experiments assessing the ability of the antisera to inhibit adhesion of IEs to ICAM-1 under flow corroborated these findings. Thus, both the above antisera (M6pep and M6pep/M9pep) significantly inhibited adhesion of IEs expressing the DBLβ_motif-containing PfEMP1 proteins PFD1235w (Fig. 5B; expressing M6 native protein) and HB3VAR03 (Fig. 5C; expressing M8 native protein), but had no effect on IEs expressing IT4VAR13 (expressing N27 native protein), which does not contain a DBLβ_motif domain (Fig. 5D). Furthermore, the single- and dual-peptide antisera yielded immunofluorescence patterns typical of IgG reacting with IE surface-expressed PfEMP1 when tested against IEs expressing either HB3VAR03 or PFD1235w, but did not label IEs expressing IT4VAR13 (Fig. 5E). In contrast, the IEs expressing IT4VAR13were labeled by an IT4VAR13-specific antiserum, but not by the single- and dual-peptide antisera (Fig. 5E).

Finally, we assessed the ability of erythrocytes infected by 33 primary *P. falciparum* isolates from Ghana (N=14) and Tanzania (N=19) to adhere to ICAM-1 under flow. We also tested the ability of pooled rat anti-serum to M6pep and M9pep to inhibit adhesion of ICAM-1-adhering isolates. Twenty-two of the isolates (three from children with uncomplicated malaria, 14 from patients with severe malaria, and five from children with cerebral malaria) showed adhesion of IEs to ICAM-1 (Fig. 6A). Adhesion of eleven of these isolates (one from a child with uncomplicated

malaria, six from children with severe malaria, and four from children with cerebral malaria) was inhibited (>25%) by the anti-peptide serum pool (Fig. 6B).

We conclude that immunization with linear peptides that represent only the ICAM-1-binding region of specific DBLβ_motif domains can induce cross-reactive antibodies that are capable of inhibiting the binding of ICAM-1 to a range of recombinant and native, IE-expressed DBLβ_motif domains. Importantly, the motif antibody inhibits binding of four of five *P. falciparum* isolates from cerebral malaria patients.

343 **DISCUSSION**

344 Cerebral malaria (CM) is one of the most severe complications of *P. falciparum* malaria, and
345 a leading cause of mortality (reviewed in ref. 50). PfEMP1-mediated adhesion of IEs to the
346 endothelial receptors ICAM-1 and EPCR have both repeatedly been implicated in the pathogenesis
347 of severe malaria (6, 25, 33). However, a specific and direct link to the development of CM has
348 been missing until recently, when we identified a sequence motif in PfEMP1 proteins associated
349 specifically with the development of CM (33). This ICAM-1-binding motif (DBLβ_motif; Fig. 1) is
350 found in some Group A PfEMP1 proteins (15), immediately downstream of an EPCR-binding
351 CIDRα domain (25). In the present study, we set out to study the acquisition of DBLβ_motif-
352 specific IgG following natural exposure, and whether DBLβ_motif-specific antibodies induced by
353 vaccination are cross-reactive and inhibit adhesion to ICAM-1.

354 In areas with stable transmission of these parasites, substantial protective immunity to malaria
355 is acquired during childhood, first to severe complications and later to clinical disease. As a
356 consequence, adults are largely protected from malaria in such areas, although sterile immunity is
357 rarely, if ever achieved. This sequence appears to be the consequence of an ordered acquisition of
358 antibodies to a relatively conserved set of PfEMP1 proteins associated with severe disease, followed
359 by antibodies to a large and diverse set of PfEMP1 proteins associated with uncomplicated malaria
360 and asymptomatic parasitemia. Where transmission is very intense, serious and fatal malaria
361 episodes are markedly concentrated during the first few years of life, mainly as severe malarial
362 anemia. CM, in contrast, is rare (51). Where endemicity is lower, CM tends to be seen more often,
363 but mainly among children some years older than those that succumb to severe malarial anemia.
364 Together these findings suggest that discrete PfEMP1 subsets are involved in severe malaria with
365 and without cerebral involvement, and perhaps even that toddlers are relatively resistant to CM for
366 non-immunologic reasons. This fits our demonstration here that although DBLβ domains are

generally immunogenic following natural exposure, acquisition of DBLβ_motif-specific IgG occurs later than DBLβ_non-motif-specific IgG (Fig. 2) and coincides with the age bracket where the incidence of CM peaks under transmission intensities comparable to our study area (52, 53).

The clinical significance of acquisition of PfEMP1-specific antibodies is thought to involve their ability to interfere with sequestration of IEs in various tissues (15, 54). A particularly thoroughly investigated example is the role of anti-adhesion antibodies in acquisition of protective immunity to placental *P. falciparum* malaria, caused by accumulation of IEs in the intervillous spaces (55, 56). Placental IE sequestration is mediated by a particular group of PfEMP1 (VAR2CSA) binding to oncofetal chondroitin sulfate A (57, 58), and clinical trials of vaccines based on the VAR2CSA adhesive epitope and aimed to protect against this important cause of prenatal and infant morbidity and mortality are currently under way. It appears that DBLβ_motif-specific IgG can inhibit IE adhesion to ICAM-1 in a similar way (Fig. 3A and (15, 33)). Here, we demonstrate that this inhibition can be mediated by genuinely cross-reactive antibodies, as opposed to a broad repertoire of IgG species each with narrow specificity for a single or very few DBLβ_motif sequences (Fig. 3). This finding is of significance, since naturally acquired protection from malaria is generally believed to be the consequence of accumulation of a broad repertoire of a large number of antibody specificities (9, 12, 59). Such broadly reactive IgG can inhibit adhesion of erythrocytes infected with parasites isolated from patients with severe malaria (six of 13 isolates) and cerebral malaria (four of five isolates) under physiologic flow conditions (Fig. 6). Furthermore, such IgG can be induced by peptides (M6pep and M9pep) representing just the core element of the DBLβ_motif that mediates the binding to ICAM-1 (Fig. 5 and Fig. 6).

Approximately half the children with acute *P. falciparum* malaria in our study had severe disease, according to the WHO criteria (60), but we did not observe any significant differences in plasma levels of DBLβ_motif-specific IgG in children with and without severe disease. This may

be related to the fact that, due to the low prevalence in the area (Table 1 and Table 2). It is doubtful that a relationship between DBLβ_motif-specific IgG and clinical presentation of acutely ill malaria patients would be apparent even if we had been able to include plasma samples from CM patients, due to the unavoidable variation in time between infection and presentation to hospital. It is plausible that only very large, and preferably longitudinal, studies would have the power required to document such relationships in semi-immune, naturally infected individuals.

In conclusion, our study demonstrates that CM-related DBLβ domains are immunogenic following natural exposure, and that acquisition of DBLβ_domain-specific IgG coincides with the age where CM has its peak prevalence in areas of moderate but stable *P. falciparum* transmission. Furthermore, we show that immunization with such domains in addition to peptides representing the minimal ICAM-1 binding region can induce IgG that can inhibit PfEMP1 binding to ICAM-1 and neutralize IE adhesion under physiologic flow. Importantly, these antibodies broadly neutralized adhesion of erythrocytes infected by parasites isolated from four of five children with cerebral malaria. Together, these findings raise hopes that development of a vaccine specifically against CM may be possible, despite the notorious polymorphism and intra-clonal diversity of the PfEMP1 family (recently reviewed in refs. 3, 61).

Acknowledgments

We thank all donors for their contribution of samples. We acknowledge the technical support of field teams in Ghana and Tanzania. We thank Mette Ulla Madsen and Maiken Howning Visti for excellent technical assistance.

Funding

This work was supported by Novo Nordisk Fonden [grant numbers NNF13OC0006249 and NNF15OC0017654], Lundbeckfonden [grant number R180-2014-3098], The Consultative Committee for Development Research [grant number DFC 12-081RH], the Danish Council for Independent Research [grant numbers 4004-00032 and 4183-00539], and Rigshospitalet [grant number R102-A4174]. The funders had no role in the study design, data collection and interpretation, or the decision to submit the work for publication.

Figure legends

Figure 1.

Sequence logo showing the ICAM-1 binding motif (as defined in (33)) of the DBLβ domains 1-15 used in the present study (Table 3). Residues that are critical for the direct interaction with ICAM-1 (red triangles) or for the architecture of the ICAM-1 binding (white triangles) are indicated. The Group A PfEMP1 ICAM-1 binding motif was identified by Lennartz *et al.* (33).

Figure 2

Plasma levels of IgG with specificity for *P. falciparum* DBLβ domains. Samples were obtained from 79 Ghanaian children with either severe (▲) or non-severe *P. falciparum* malaria. *A,* Levels (ELISA units; EU) in plasma from individual children (columns) of IgG specific for individual group A DBLβ domains (rows) containing (DBLβ_motif; M1-M12; M14-M15) or not containing (DBLβ_non-motif; N20-N32; lower half) the ICAM-1-binding motif identified by Lennartz *et al.* (33). Shading indicate IgG level score: Black (4: >100 EU), dark gray (3: 76-100 EU), gray (2: 51-75 EU), light gray (1: 26-50 EU), and white (0-25 EU). The DBLβ domain numbers correspond to the numbers in Table 3. Danish controls (n= 25) did not react with any of the domains (data not shown). *B,* The means of IgG level scores (defined as in *A*) of individual DBLβ domains that contain (Motif; ○) or do not contain (Non-motif; ●) the ICAM-1-binding motif. Error bars indicate 95% confidence intervals. DBLβ domain numbering as in *A*. *C*, The means of IgG level scores (defined as in *A*) of individual children for IgG specific for DBLβ_motif (○) and DBLβ_non-motif (●) domains. The statistical significance (Mann-Whitney rank-sum test) of pairwise comparisons is shown along the top of the panel.

Figure 3

Ability of DBLβ_motif-specific IgG to inhibit binding of ICAM-1 to DBLβ domains. *A,* Inhibition of ICAM-1 binding by pooled immune plasma from 10 of the study children (Pool). *B,* Rat anti-sera raised against DBLβ_motif antigens tested against recombinant proteins containing (M1-M7, M9-M15) or not containing (N27, N33) DBLβ_motif. Shading indicates degree of inhibition: black (>75%), dark gray (50-75%), gray (20-50%), and white (<20%). The DBLβ domain numbers and antiserum specificities correspond to the numbers in Table 3. Data using a pool of rat-anti-sera (M1, M6, M9, M10) are also shown (Pool). Anti-sera marked with asterisks were affinity-purified on a peptide (M6pep) representing the binding motif in PFD1235w_DBLβ_D4 prior to assaying. Three independent experiments were done (three technical replicates/assay). A sequence-distance tree illustrating the relatedness of the different domains is shown along the left edge of the panel.

Figure 4

DBLβ-specific antibody-mediated inhibition of adhesion of IEs to ICAM-1 under physiologic shear stress, relative to control without antibody (None). *A,* IEs expressing PFD1235w. *B,* IEs expressing HB3VAR03. *C,* IEs expressing IT4VAR13. The specificities of the DBLβ antibodies correspond to the numbers in Table 3. Anti-serum marked with asterisk was affinity-purified on a peptide (M6pep) representing the binding motif in PFD1235w_DBLβ_D4 prior to assaying. An ICAM-1-specific neutralizing antibody (ICAM-1) and an irrelevant rat anti-IgG (https://www.sigmaaldrich.com) were included as positive and negative controls, respectively. Fewer than 0.25 IEs/mm^2 bound to uncoated channels. Means (bars) and standard deviations (error bars) of at least three independent experiments in triplicates are shown. Statistically significant reductions relative to adhesion in the absence of antibody (–) are indicated above the bars (**, P(F)<0.01; ***, P(F)<0.001). Refer to Table S1 for raw data.

Figure 3

Ability of DBLβ_motif-specific IgG to inhibit binding of ICAM-1 to DBLβ domains. *A,* Inhibition of ICAM-1 binding by pooled immune plasma from 10 of the study children (Pool). *B,* Rat anti-sera raised against DBLβ_motif antigens tested against recombinant proteins containing (M1-M7, M9-M15) or not containing (N27, N33) DBLβ_motif. Shading indicates degree of inhibition: black (>75%), dark gray (50-75%), gray (20-50%), and white (<20%). The DBLβ domain numbers and antiserum specificities correspond to the numbers in Table 3. Data using a pool of rat-anti-sera (M1, M6, M9, M10) are also shown (Pool). Anti-sera marked with asterisks were affinity-purified on a peptide (M6pep) representing the binding motif in PFD1235w_DBLβ_D4 prior to assaying. Three independent experiments were done (three technical replicates/assay). A sequence-distance tree illustrating the relatedness of the different domains is shown along the left edge of the panel.

Figure 4

DBLβ-specific antibody-mediated inhibition of adhesion of IEs to ICAM-1 under physiologic shear stress, relative to control without antibody (None). *A,* IEs expressing PFD1235w. *B,* IEs expressing HB3VAR03. *C,* IEs expressing IT4VAR13. The specificities of the DBLβ antibodies correspond to the numbers in Table 3. Anti-serum marked with asterisk was affinity-purified on a peptide (M6pep) representing the binding motif in PFD1235w_DBLβ_D4 prior to assaying. An ICAM-1-specific neutralizing antibody (ICAM-1) and an irrelevant rat anti-IgG (https://www.sigmaaldrich.com) were included as positive and negative controls, respectively. Fewer than 0.25 IEs/mm^2 bound to uncoated channels. Means (bars) and standard deviations (error bars) of at least three independent experiments in triplicates are shown. Statistically significant reductions relative to adhesion in the absence of antibody (–) are indicated above the bars (**, P(F)<0.01; ***, P(F)<0.001). Refer to Table S1 for raw data.

Figure 5

Ability of rat antisera to the ICAM-1-binding motif in DBLβ_motif domains to inhibit binding of recombinant DBLβ domains to ICAM-1. *A,* Anti-sera from rats immunized with M6pep or with both M6pep and M9pep tested against recombinant DBLβ_motif domains (M2-M7, M9, M11-M13) and DBLβ_non-motif domains (N27, N33). Shading, DBLβ domain numbers, and antiserum specificities as in Fig. 3B. *B-D,* Inhibition by the same anti-sera of ICAM-1-specific adhesion of PFD1235w^{+} IEs (*B*), HB3VAR03^{+} IEs (*C*), and IT4VAR13^{+} IEs (*D*) under physiologic shear stress. Statistical significance of reductions is indicated as in Fig. 4. Three independent experiments were done (with three technical replicates in each). Fewer than 0.25 IEs/mm^2 were observed bound to uncoated channels (*E*), Immunofluorescence of representative IEs with surface expression of PFD1235w (top row), HB3VAR03 (center row), and IT4VAR13 (bottom row) and labeled by sera from rats immunized with M6pep only (left column) or with both M6pep and M9pep (center column), or by a rat anti-serum to N27 (right column). Refer to Table S2 for raw data.

Figure 6

ICAM-1-specific adhesion of erythrocytes infected by patient *P. falciparum* isolates, and inhibition of ICAM-1-adhering IEs by M6pep/M9pep-specific antibody. *A*, Adhesion of 33 patient isolates to ICAM-1 under physiologic flow. *B*, Antibody-mediated inhibition (>25%) of ICAM-1-specific IE adhesion among the 22 patient isolates adhering (≥ 10 adherent IEs/mm^2) to ICAM-1 under physiologic flow. The isolates were tested in one experiment with five technical replicates. Fewer than 0.25 IEs/mm^2 bound to uncoated channels. Isolates from patients with uncomplicated malaria (○), cerebral malaria (▲), and non-cerebral severe disease (●) are indicated in both panels.

485 **REFERENCES**

486 1. Anonymous. 2016. World Health Organization. World Malaria Report 2016. Organization
487 WH, Geneva.
488 2. Cowman AF, Healer J, Marapana D, Marsh K. 2016. Malaria: biology and disease. Cell
489 167:610-624.
490 3. Hviid L, Jensen AT. 2015. PfEMP1 - a parasite protein family of key importance in
491 *Plasmodium falciparum* malaria immunity and pathogenesis. Adv Parasitol 88:51-84.
492 4. Kraemer SM, Smith JD. 2003. Evidence for the importance of genetic structuring to the
493 structural and functional specialization of the *Plasmodium falciparum var* gene family. Mol
494 Microbiol 50:1527-1538.
495 5. Lavstsen T, Salanti A, Jensen ATR, Arnot DE, Theander TG. 2003. Sub-grouping of
496 *Plasmodium falciparum* 3D7 var genes based on sequence analysis of coding and non-
497 coding regions. Malar J 2:27.
498 6. Jensen ATR, Magistrado PA, Sharp S, Joergensen L, Lavstsen T, Chiucchiuini A, Salanti A,
499 Vestergaard LS, Lusingu JP, Hermsen R, Sauerwein R, Christensen J, Nielsen MA, Hviid L,
500 Sutherland C, Staalsoe T, Theander TG. 2004. *Plasmodium falciparum* associated with
501 severe childhood malaria preferentially expresses PfEMP1 encoded by Group A *var* genes. J
502 Exp Med 199:1179-1190.
503 7. Rottmann M, Lavstsen T, Mugasa JP, Kaestli M, Jensen ATR, Müller D, Theander T, Beck
504 H-P. 2006. Differential expression of *var* gene groups is associated with morbidity caused
505 by *Plasmodium falciparum* infection in Tanzanian children. Infect Immun 74:3904-3911.
506 8. Bull PC, Kortok M, Kai O, Ndungu F, Ross A, Lowe BS, Newbold CI, Marsh K. 2000.
507 *Plasmodium falciparum*-infected erythrocytes: agglutination by diverse Kenyan plasma is
508 associated with severe disease and young host age. J Infect Dis 182:252-259.

9. Nielsen MA, Staalsoe T, Kurtzhals JAL, Goka BQ, Dodoo D, Alifrangis M, Theander TG, Akanmori BD, Hviid L. 2002. *Plasmodium falciparum* variant surface antigen expression varies between isolates causing severe and non-severe malaria and is modified by acquired immunity. J Immunol 168:3444-3450.

10. Gupta S, Snow RW, Donnelly CA, Marsh K, Newbold C. 1999. Immunity to non-cerebral severe malaria is acquired after one or two infections. Nat Med 5:340-343.

11. Warimwe G, Keane TM, Fegan G, Musyoki JN, Newton CRJC, Pain A, Berriman M, Marsh K, Bull PC. 2009. *Plasmodium falciparum var* gene expression is modified by host immunity. PNAS 106:21801-21806.

12. Cham GK, Turner L, Kurtis JD, Mutabingwa T, Fried M, Jensen AT, Lavstsen T, Hviid L, Duffy PE, Theander TG. 2010. Hierarchical, domain type-specific acquisition of antibodies to *Plasmodium falciparum* erythrocyte membrane protein 1 in Tanzanian children. Infect Immun 78:4653-4659.

13. Rask TS, Hansen DA, Theander TG, Pedersen AG, Lavstsen T. 2010. *Plasmodium falciparum* erythrocyte membrane protein 1 diversity in seven genomes - divide and conquer. PLoS Comput Biol 6:e1000933.

14. Lavstsen T, Turner L, Saguti F, Magistrado P, Rask TS, Jespersen JS, Wang CW, Berger SS, Baraka V, Marquard AM, Seguin-Orlando A, Willerslev E, Gilbert MT, Lusingu J, Theander TG. 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:E1791-E1800.

15. Bengtsson A, Joergensen L, Rask TS, Olsen RW, Andersen MA, Turner L, Theander TG, Hviid L, Higgins MK, Craig A, Brown A, Jensen AT. 2013. A novel domain cassette

identifies *Plasmodium falciparum* PfEMP1 proteins binding ICAM-1 and is a target of cross-reactive, adhesion-inhibitory antibodies. J Immunol 190:240-249.

16. Berger SS, Turner L, Wang CW, Petersen JE, Kraft M, Lusingu JP, Mmbando B, Marquard AM, Bengtsson DB, Hviid L, Nielsen MA, Theander TG, Lavstsen T. 2013. *Plasmodium falciparum* expressing domain cassette 5 type PfEMP1 (DC5-PfEMP1) bind PECAM1. PLoS One 8:e69117.

17. Avril M, Tripathi AK, Brazier AJ, Andisi C, Janes JH, Soma VL, Sullivan DJ, Jr., Bull PC, Stins MF, Smith JD. 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:E1782-E1790.

18. Claessens A, Adams Y, Ghumra A, Lindergard G, Buchan CC, Andisi C, Bull PC, Mok S, Gupta AP, Wang CW, Turner L, Arman M, Raza A, Bozdech Z, Rowe JA. 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:E1772-E1781.

19. Lusingu JP, Jensen AT, Vestergaard LS, Minja DT, Dalgaard MB, Gesase S, Mmbando BP, Kitua AY, Lemnge MM, Cavanagh D, Hviid L, Theander TG. 2006. Levels of plasma immunoglobulin G with specificity against the cysteine-rich interdomain regions of a semiconserved *Plasmodium falciparum* erythrocyte membrane protein 1, VAR4, predict protection against malarial anemia and febrile episodes. Infect Immun 74:2867-2875.

20. Turner GDH, Morrison H, Jones M, Davis TME, Looareesuwan S, Buley ID, Gatter KC, Newbold CI, Pukrittayakamee S, Nagachinta B, White NJ, Berendt AR. 1994. An immunohistochemical study of the pathology of fatal malaria: evidence for widespread endothelial activation and a potential role for intercellular adhesion molecule-1 in cerebral sequestration. Am J Pathol 145:1057-1069.

21. 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 57:389-398.

22. Rogerson SJ, Tembenu R, Dobaño C, Plitt S, Taylor TE, Molyneux ME. 1999. Cytoadherence characteristics of *Plasmodium falciparum*-infected erythrocytes from Malawian children with severe and uncomplicated malaria. Am J Trop Med Hyg 61:467-472.

23. 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 69:5849-5856.

24. Ochola LB, Siddondo BR, Ocholla H, Nkya S, Kimani EN, Williams TN, Makale JO, Liljander A, Urban BC, Bull PC, Szestak T, Marsh K, Craig AG. 2011. Specific receptor usage in *Plasmodium falciparum* cytoadherence is associated with disease outcome. PLoS One 6:e14741.

25. Turner L, Lavstsen T, Berger SS, Wang CW, Petersen JE, Avril M, Brazier AJ, Freeth J, Jespersen JS, Nielsen MA, Magistrado P, Lusingu J, Smith JD, Higgins MK, Theander TG. 2013. Severe malaria is associated with parasite binding to endothelial protein C receptor. Nature 498:502-505.

26. Moxon CA, Wassmer SC, Milner DA, Jr., Chisala NV, Taylor TE, Seydel KB, Molyneux ME, Faragher B, Esmon CT, Downey C, Toh CH, Craig AG, Heyderman RS. 2013. Loss of endothelial protein C receptors links coagulation and inflammation to parasite sequestration in cerebral malaria in African children. Blood 122:842-851.

27. Jespersen JS, Wang CW, Mkumbaye SI, Minja DT, Petersen B, Turner L, Petersen JE, Lusingu JP, Theander TG, Lavstsen T. 2016. *Plasmodium falciparum var* genes expressed in children with severe malaria encode CIDRa1 domains. EMBO Mol Med 8:839-850.

28. Mkumbaye SI, Wang CW, Lyimo E, Jespersen JS, Manjurano A, Mosha J, Kavishe RA, Mwakalinga SB, Minja DT, Lusingu JP, Theander TG, Lavstsen T. 2017. The severity of *Plasmodium falciparum* infection is associated with transcript levels of *var* genes encoding EPCR-binding PfEMP1. Infect Immun 85:e00841-16.

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

30. Schuldt K, Ehmen C, Evans J, May J, Ansong D, Sievertsen J, Muntau B, Ruge G, Agbenyega T, Horstmann RD. 2014. Endothelial protein C receptor gene variants not associated with severe malaria in Ghanaian children. PLoS One 9:e115770.

31. Hansson HH, Turner L, Moller L, Wang CW, Minja DT, Gesase S, Mmbando B, Bygbjerg IC, Theander TG, Lusingu JP, Alifrangis M, Lavstsen T. 2015. Haplotypes of the endothelial protein C receptor (EPCR) gene are not associated with severe malaria in Tanzania. Malar J 14:474.

32. Avril M, Bernabeu M, Benjamin M, Brazier AJ, Smith JD. 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:e00615-16.

33. Lennartz F, Adams Y, Bengtsson A, Olsen RW, Turner L, Ndam NT, Ecklu-Mensah G, Moussiliou A, Ofori MF, Gamain B, Lusingu JP, Petersen JE, Wang CW, Nunes-Silva S, Jespersen JS, Lau CK, Theander TG, Lavstsen T, Hviid L, Higgins MK, Jensen AT. 2017.

Structure-guided identification of a family of dual receptor-binding PfEMP1 that is associated with cerebral malaria. Cell Host Microbe 21:403-414.

34. Stevenson L, Laursen E, Cowan GJ, Bandoh B, Barfod L, Cavanagh DR, Andersen GR, Hviid L. 2015. a_2-macroglobulin can crosslink multiple *Plasmodium falciparum* erythrocyte membrane protein 1 (PfEMP1) molecules and may facilitate adhesion of parasitized erythrocytes. PLoS Pathog 11:e1005022.

35. Lavstsen T, Turner L, Saguti F, Magistrado P, Rask TS, Jespersen JS, Wang CW, Berger SS, Baraka V, Marquard AM, Seguin-Orlando A, Willerslev E, Gilbert MT, Lusingu J, Theander TG. 2012. Plasmodium falciparum erythrocyte membrane protein 1 domain cassettes 8 and 13 are associated with severe malaria in children. ProcNatlAcadSciUSA 109:E1791-E1800.

36. Lusingu JP, Vestergaard LS, Mmbando BP, Drakeley CJ, Jones C, Akida J, Savaeli ZX, Kitua AY, Lemnge MM, Theander TG. 2004. Malaria morbidity and immunity among residents of villages with different *Plasmodium falciparum* transmission intensity in North-Eastern Tanzania. Malar J 3:26.

37. Bengtsson A, Joergensen L, Barbati ZR, Craig A, Hviid L, Jensen AT. 2013. Transfected HEK293 cells expressing functional recombinant intercellular adhesion molecule 1 (ICAM-1) - a receptor associated with severe *Plasmodium falciparum* malaria. PLoS One 8:e69999.

38. Joergensen L, Bengtsson DC, Bengtsson A, Ronander E, Berger SS, Turner L, Dalgaard MB, Cham GK, Victor ME, Lavstsen T, Theander TG, Arnot DE, Jensen AT. 2010. Surface co-expression of two different PfEMP1 antigens on single *Plasmodium falciparum*-infected erythrocytes facilitates binding to ICAM1 and PECAM1. PLoS Pathog 6:e1001083.

39. Jensen ATR, Zornig HD, Buhmann C, Salanti A, Koram KA, Riley EM, Theander TG, Hviid L, Staalsoe T. 2003. Lack of gender-specific antibody recognition of products from

domains of a *var* gene implicated in pregnancy-associated *Plasmodium falciparum* malaria. Infect Immun 71:4193-4196.

40. Barfod L, Dalgaard MB, Pleman ST, Ofori MF, Pleass RJ, Hviid L. 2011. Evasion of immunity to *Plasmodium falciparum* malaria by IgM masking of protective IgG epitopes in infected erythrocyte surface-exposed PfEMP1. Proc Natl Acad Sci U S A 108:12485-12490.

41. Staalsoe T, Giha HA, Dodoo D, Theander TG, Hviid L. 1999. Detection of antibodies to variant antigens on *Plasmodium falciparum* infected erythrocytes by flow cytometry. Cytometry 35:329-336.

42. Cranmer SL, Magowan C, Liang J, Coppel RL, Cooke BM. 1997. An alternative to serum for cultivation of *Plasmodium falciparum in vitro*. Transactions of the Royal Society for Tropical Medicine and Hygiene 91:363-365.

43. Snounou G, Zhu XP, Siripoon N, Jarra W, Thaithong S, Brown KN, Viriyakosol S. 1999. Biased distribution of *msp1* and *msp2* allelic variants in *Plasmodium falciparum* populations in Thailand. Trans R Soc Trop Med Hyg 93:369-374.

44. Lennartz F, Bengtsson A, Olsen RW, Joergensen L, Brown A, Remy L, Man P, Forest E, Barfod LK, Adams Y, Higgins MK, Jensen AT. 2015. Mapping the binding site of a cross-reactive *Plasmodium falciparum* PfEMP1 monoclonal antibody inhibitory of ICAM-1 binding. J Immunol 195:3273-3283.

45. Ghumra A, Semblat J-P, McIntosh RS, Raza A, Rasmussen IB, Braathen R, Johansen F-E, Sandlie I, Mongini PK, Rowe JA, Pleass RJ. 2008. Identification of residues in the Cm4 domain of polymeric IgM essential for interaction with *Plasmodium falciparum* erythrocyte membrane protein 1 (PfEMP1). J Immunol 181:1988-2000.

46. Edgar RC. 2004. MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Res 32:1792-1797.

649 47. Tamura K, Dudley J, Nei M, Kumar S. 2007. MEGA4: Molecular evolutionary genetics
650 analysis (MEGA) software version 4.0. Mol Biol Evol 24:1596-1599.
651 48. Crooks GE, Hon G, Chandonia JM, Brenner SE. 2004. WebLogo: a sequence logo
652 generator. Genome Res 14:1188-1190.
653 49. Barfod L, Dobrilovic T, Magistrado P, Khunrae P, Viwami F, Bruun J, Dahlbäck M,
654 Bernasconi NL, Fried M, John D, Duffy PE, Salanti A, Lanzavecchia A, Lim CT, Ndam
655 NT, Higgins MK, Hviid L. 2010. Chondroitin sulfate A-adhering *Plasmodium falciparum*-
656 infected erythrocytes express functionally important antibody epitopes shared by multiple
657 variants. J Immunol 185:7553-7561.
658 50. Storm J, Craig AG. 2014. Pathogenesis of cerebral malaria - a combination of inflammation
659 and cytoadherence. Front Cell Infect Microbiol 4:100.
660 51. Snow RW, Omumbo JA, Lowe B, Molyneux CS, Obiero JO, Palmer A, Weber MW, Pinder
661 M, Nahlen B, Obonyo C, Newbold C, Gupta S, Marsh K. 1997. Relation between severe
662 malaria morbidity in children and level of *Plasmodium falciparum* transmission in Africa.
663 Lancet 349:1650-1654.
664 52. Struik SS, Riley EM. 2004. Does malaria suffer from lack of memory? Immunol Rev
665 201:268-290.
666 53. Reyburn H, Mbatia R, Drakeley C, Bruce J, Carneiro I, Olomi R, Cox J, Nkya WM,
667 Lemnge M, Greenwood BM, Riley EM. 2005. Association of transmission intensity and age
668 with clinical manifestations and case fatality of severe *Plasmodium falciparum* malaria.
669 JAMA 293:1461-1470.
670 54. Oleinikov AV, Amos E, Frye IT, Rossnagle E, Mutabingwa TK, Fried M, Duffy PE. 2009.
671 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:e1000386.

55. Fried M, Nosten F, Brockman A, Brabin BT, Duffy PE. 1998. Maternal antibodies block malaria. Nature 395:851-852.

56. Salanti A, Dahlbäck M, Turner L, Nielsen MA, Barfod L, Magistrado P, Jensen ATR, Lavstsen T, Ofori MF, Marsh K, Hviid L, Theander TG. 2004. Evidence for the involvement of VAR2CSA in pregnancy-associated malaria. J Exp Med 200:1197-1203.

57. Salanti A, Staalsoe T, Lavstsen T, Jensen ATR, Sowa MPK, Arnot DE, Hviid L, Theander TG. 2003. Selective upregulation of a single distinctly structured *var* gene in CSA-adhering *Plasmodium falciparum* involved in pregnancy-associated malaria. Mol Microbiol 49:179-191.

58. Clausen TM, Christoffersen S, Dahlback M, Langkilde AE, Jensen KE, Resende M, Agerbak MO, Andersen D, Berisha B, Ditlev SB, Pinto VV, Nielsen MA, Theander TG, Larsen S, Salanti A. 2012. Structural and functional insight into how the *Plasmodium falciparum* VAR2CSA protein mediates binding to chondroitin sulfate A in placental malaria. J Biol Chem 287:23332-23345.

59. Bull PC, Lowe BS, Kortok M, Molyneux CS, Newbold CI, Marsh K. 1998. Parasite antigens on the infected red cell are targets for naturally acquired immunity to malaria. Nat Med 4:358-360.

60. Warrell DA, Molyneux ME, Beales PF. 1990. Severe and complicated malaria. Second edition. Transactions of the Royal Society for Tropical Medicine and Hygiene 84 (suppl. 2):1-65.

61. Bull PC, Abdi AI. 2016. The role of PfEMP1 as targets of naturally acquired immunity to childhood malaria: prospects for a vaccine. Parasitology 143:171-186.

696 62. Howell DP, Levin EA, Springer AL, Kraemer SM, Phippard DJ, Schief WR, Smith JD.
697 2008. Mapping a common interaction site used by *Plasmodium falciparum* Duffy binding-
698 like domains to bind diverse host receptors. Mol Microbiol 67:78-87.
699

700 **Table 1: Clinical characteristic of Ghanaian study participants contributing plasma**

	Severe malaria (N=35)				Uncomplicated malaria (N=44)			
Age group (number)	1-2 (n=16)	3-4 (n=9)	5-6 (n=6)	>6 (n=4)	1-2 (n=8)	3-4 (n=15)	5-6 (n=8)	>6 (n=13)
Age years[1]	2.2 (1.9;2.7)	3.4 (3.2;4.3)	5.5 (5.3;6.6)	7.8 (7.5;8.2)	2.5 (1.8;2.7)	3.9 (3.4;4.4)	5.9 (5.3;6.9)	9.2 (8.1;10.8)
Blantyre coma score[1]	5.0 (5.0;5.0)	5.0 (4.3;5.0)	5.0 (4.3;5.0)	4,0 (3.0;5.0)	5.0 (5.0;5.0)	5.0 (5.0;5.0)	5.0 (5.0;5.0)	5.0 (5.0;5.0)
Haemoglobin (g/dl)[1]	9.6 (5.6;10.8)	6.3 (3.1;9.6)	10.3 (8.8;10.9)	10.8 (7.0;12.0)	9.6 (8.9;10.3)	9.6 (6.9;10.8)	9.5 (8,4;11.9)	11.4 (10.6;11.7)
Parasites per µl (x1000)[1]	41.5 (14.3;171.0)	147.3 (4.3; 210.8)	123.2 (2.9; 204.9)	114.5 (56.3; 152.6)	8.3 (0.4;26.0)	18.0 (6.3; 62.0)	19.7 (3.2;71.2)	14.7 (1.6;109.9)

701 [1]Median (25%;75%). None of the participants donating plasma was diagnosed with cerebral malaria.

Table 2: Clinical characteristics of Ghanaian and Tanzanian study participants contributing *P. falciparum* parasite isolates

	Severe malaria (N=24)				Uncomplicated malaria (N=9)			
Age group (number)	<1 (n=3)	1-2 (n=11)	3-4 (n=7)	≥5 (n=3)	<1 (n=1)	1-2 (n=2)	3-4 (n=4)	≥5 (n=2)
Age years[1]	0.9 (0.89; 0.95)	2.0 (1.67;2.53)	4.01 (3.47; 4.78)	7.4 (5.56;7.68)	0.36	2.50 2.76	3.6 (3.07; 4.58)	6.4 11.3
Blantyre coma score[1]	1.0 (0.0;5.0)	5.0 (2.0;5.0)	5.0 (2.0;5.0)	3.0 (2.0;3.0)	5.0	5.0	5.0 (5.0;5.0)	5.0 5.0
Hemoglobin (g/dl)[1]	7.8 (4.2;8.2)	4.7 (4.4;6.1)	8.6 (3.8;10.8)	10.5 (6.1;11.8)	11.8	12.0 12.3	8.6 (6.9;10.2)	12.5 11.7
Parasites per µl (x1000)[1]	64.8 (4.0; 165.4)	74.8 (33.2; 194.5)	182.9 (42.2; 456.2)	63.3 (49.2;77.5)	91.0	77.5 70.5	58.9 (18.7;133.4)	87.4 13.7

[1]Median (25%;75%). The severe malaria includes patients with cerebral malaria (N=7), severe malarial anemia (N=11) and hyperparasitaemia, multiple convulsions and/or respiratory distress (N=9).

705 **Table 3. Recombinant proteins used in the study**

	ID	Genome	PfEMP1	Domain sub-type [1]	Binds ICAM-1 [2]	Group [3]	Gene source [9]
DBLβ_motif domains[13]	M1	3D7	PF11_0521[4]	DBLβ3_D4	Yes[10]	A	
	M2	BM048	JF712902	DBLβ3_D4	Yes[10]	A	
	M3	BM066	JF712903	DBLβ3_D4	Yes[10]	A	
	M4	BM021	JF712900	DBLβ3_D4	Yes[10]	A	
	M5	BM057	JN037695	DBLβ3_D4	Yes[10]	A	
	M6	3D7	PFD1235w[5]	DBLβ3_D4	Yes[10]	A	
	M7	MN35	KJ866957	DBLβ3_D4	Yes[10]	A	
	M8	HB3	VAR03	DBLβ3_D4	Yes[10]	A	
	M9	Dd2	VAR32[6]	DBLβ1_D4	Yes[10]	A	
	M10	MN56	KM364031	DBLβ1_D4	Yes[10]	A	
	M11	A4395	KJ866958	DBLβ3	Yes[10]	A	
	M12	1914	AFJ66668	DBLβ1_D4	Yes[10]	A	
	M13	BM028	JF712901	DBLβ3_D4	Yes[10]	A	
	M14	-	KM364033	DBLβ3	Yes[10]	A	
	M15	MN062	KF984156	DBLβ1_D4	Yes[10]	A	
DBLβ_non-motif domains[13]	N20		CDO62031		No[11]	A	Synthetic gene (https://www.eurofinsgenomics.eu/)
	N21		CDO61797		No[11]	A	Synthetic gene (https://www.eurofinsgenomics.eu/)
	N22		CDO63496		No[11]	A	Synthetic gene (https://www.eurofinsgenomics.eu/)
	N23	Dd2	VAR25	DBLβ11_D4	No[10]	A	
	N24	HB3	VAR1CSA	DBLβ11_D4	No[10]	A	
	N25	3D7	PF13_0003	DBLβ9_D8	No[10]	A	
	N26	A4393	KJ866959	DBLβ3	No[10]	A	
	N27	IT4	IT4VAR13[7]	DBLβ3_D4	Yes[10+12]	B	
	N28	1983	JQ691647	DBLβ3_D4	No[10]	A	

N29	MN35	KM364034	DBLβ6	No[10]	A	
N30	Dd2	VAR52	DBLβ7_D4	No[10]	A	
N31	HB3	VAR01	DBLβ7_D4	No[10]	A	
N32	1983	JQ691649	DBLβ6_D4	No[10]	A	
N33	IT4	IT4VAR16[8]	DBLβ5_D4	Yes[10+12]	B	Genomic DNA using forward/reverse primers: 5'-ATCCCGGGTGTGCTGAACCTAATGGTAG-3'/ 5'-ATGCGGCCGCTACAAGCACACGCATCATC-3'

[1] Nomenclature as described in (15). [2] Yes indicates DBLβ_motif domains that bind ICAM-1 (M1-M15, N27 and N33), No indicates domains that do not bind ICAM-1 (remainder). [3] All domains were group A except for two group B DBLβ domains as indicated (N27 and N33). [4] a.k.a. PF3D7_1150400. [5] a.k.a. PF3D7_0425800. [6] a.k.a. KOB85388. [7] a.k.a. ABM88750. [8] a.k.a. AAS89259. [9] From genomic DNA, using previously described primers (15, 33), except where indicated. [10] (Data in ref. 33). [11] Unpublished data. [12] (Data in ref. 62). [13] (Data in refs. 15, 33, 62).