Antibody immunity to *Plasmodium falciparum* erythrocyte membrane protein 1 and severe malaria

Presented by

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Abstract

Plasmodium falciparum erythrocyte membrane protein 1 (PfEMP1) proteins are expressed on the surface of the infected erythrocyte (IE). These proteins mediate the adhesion of the parasite IE to host cells which could result in severe forms of malaria. PfEMP1 is a variable protein and consists of several domains. PfEMP1s are encoded by *var* genes and composed of multiple duffy binding-like (DBL) domains and cysteine-rich inter-domain region (CIDR) domains. PfEMP1s can be classified into groups based on the chromosomal location, transcriptional direction and upstream promotor regions of *var* genes, the DBL or CIDR domains expressed or the domain cassettes (DC) expressed. DCs are sets of PfEMP1 domains that usually occur together. Transcription of specific PfEMP1 subtypes and DCs have been associated with severe malaria. These proteins are important for anti-malarial immunity. Antibody immunity to the different PfEMP1 types can develop gradually with exposure with potential to protect from severe malaria. The current study investigated if antibody responses to different subsets of PfEMP1s or selections of their constituent domains differed between individuals with severe malaria and uncomplicated malaria as well as between individuals presenting with specific severe malaria syndromes. The study also investigated how such antibodies vary between disease presentation and convalescence, 1-2 months after the malaria episode.

Three aims were investigated in the present study: 1) to determine if recombinant CIDR domains are targets of antibodies in children with severe or uncomplicated malaria, 2) to determine if levels of opsonising antibodies against four parasite IE lines differed between children with severe or uncomplicated malaria and 3) to determine if the various recombinant PfEMP1 domains (transcripts of which were previously shown to be associated with severe or uncomplicated malaria in adults) are targets of antibody immunity in the same adults with severe or uncomplicated malaria as well as in children with cerebral or uncomplicated malaria. To measure antibody levels against the different PfEMP1 subtypes, unique, high-throughput multiplex assays as well as flow cytometry-based assays were employed. Antibody levels were measured in children from Papua New Guinea (PNG), adults from Papua and children from Malawi.

For aim 1, total IgG levels were measured and analysed against 35 recombinant CIDR domains in plasma collected from PNG children at hospital presentation (acute) and in convalescence following severe (n = 98) or uncomplicated malaria (n = 65), and in age-matched community controls (n = 112). At hospital presentation, antibody levels did not differ across the three severity groups for any of the CIDR domain types tested. During convalescence, antibody levels against the endothelial protein C receptor (EPCR) binding

CIDRα1 domain types were higher in older children with severe malaria compared to uncomplicated malaria. Antibodies to the EPCR-binding CIDRα1 domains also increased from presentation to convalescence for severe malaria cases, but not uncomplicated malaria. Such differences in antibody acquisition (by disease severity) or boosting of antibody levels (with time) against non-EPCR-binding CIDR domains, such as the CD36 binding domains or domains associated with rosetting, were not observed, suggesting that these variants may not be prominently expressed in these infections.

Antibodies acquired against PfEMP1 can have varied functions such as inhibiting the IE-receptor binding, opsonic phagocytosis and inhibition of rosette formation, which in turn can potentially protect from severe malaria. Using the same PNG study population as aim 1, for aim 2, levels of one type of functional antibodies -opsonising antibodies- were measured and analysed in plasma collected at acute and convalescence in children with severe (n = 163 and 158 respectively) or uncomplicated malaria (n = 101 and 99 respectively) as well as age-matched community controls (n = 235). Opsonising antibody levels against four different parasite IE expressing the entire PfEMP1 molecule, with conformations similar to the native protein and having different binding phenotypes, were measured. Opsonising antibodies against an EPCR-binding PfEMP1 were higher in children having uncomplicated malaria compared to children with severe malaria. These antibody levels were higher in children with uncomplicated malaria compared to children with severe malaria presenting with specific severe syndromes as well. The antibody levels were also boosted from presentation to convalescence for the EPCR binding IE in children with severe, but not uncomplicated, malaria. For the intercellular adhesion molecule 1 (ICAM-1) -binding PfEMP1 (which also binds to CD36), children with uncomplicated malaria showed weak evidence for boosting of antibodies from presentation to convalescence. For the rosetting IE, opsonising antibodies acquired during convalescence were higher in children with uncomplicated malaria compared to severe malaria. Antibodies against the CD36-binding PfEMP1 did not differ by disease severity and declined from presentation to convalescence in severe and uncomplicated cases.

PfEMP1 is a large multi-domain protein and for aim 3, to investigate the role of antibodies against different PfEMP1 domains, the domains (whose transcription was upregulated in severe or uncomplicated malaria cases in Papua) were first expressed using the wheat germ cell free protein synthesis systems (WGCFS). Antibody levels against 32 recombinant PfEMP1 domains thus generated were measured in acute plasma from Papuan individuals with severe (n = 28) or uncomplicated malaria (n = 35). Antibody levels against these domains were also measured in acute and convalescent plasma

from Malawian children with cerebral malaria (n = 119 and 68 respectively) or uncomplicated malaria (n = 118 and 85 respectively), and in age-matched healthy individuals (n = 101).

In the Papuan population, antibodies were higher in individuals with uncomplicated malaria compared to severe malaria against a range of PfEMP1 domains. The differences in antibody levels by disease severity against the different domains included PfEMP1 domains such as CIDRα1.6 and DBLβ3, which were previously associated with severe malaria, as well as several novel PfEMP1 domains such as CIDRα2.4, DBLζ4, and DBLδ1.

In the Malawian population, antibody levels were higher in children with cerebral malaria compared to uncomplicated malaria at presentation but not in convalescence. Antibody levels against various PfEMP1 domains were boosted from presentation to follow-up in children with uncomplicated malaria but not severe malaria. The differences in antibody levels by disease severity against the different domains for this population also included PfEMP1 domains such as CIDRα1.1 which was previously associated with severe malaria, as well as several novel PfEMP1 domains such as CIDRα2.4, DBLε3, DBLγ3, DBLζ3, DBLβ13, DBLδ7 and DBLδ1. There were some novel overlapping targets of immunity in the two populations, while some domains did not overlap as targets of immunity in the two settings, such as DBLζ4.

Thus, the current study was one of the first studies to demonstrate the EPCR-binding PfEMP1 domains or types to be important targets of antibody immunity as evidenced by differences in the antibody levels by disease severity against these domains. The present study was also one of the first to show levels of opsonising antibodies against the EPCR-binding PfEMP1 to differ by disease severity with potential to protect from severe malaria and its syndromes. Weak evidence was also found for opsonising antibodies against ICAM-1/CD36-binding PfEMP1 to be associated with uncomplicated malaria. In Papuan adults, protection from severe malaria was associated with a ‘total’ antibody response against a wide range of PfEMP1 domains whose transcripts were also upregulated in severe infections affecting the same individuals. In young Malawian children with cerebral malaria, the antibody responses against the PfEMP1 domains seem to be markers of exposure to antigens causing malaria rather than markers of immunity.

The overall conclusion for the study is that antibody immunity to multiple PfEMP1 domains on the IE may be required for protection from severe malaria and the current study has helped identify some of these PfEMP1 domains.

Declaration

This is to certify that:

1. This thesis comprises only my original work towards the PhD except where indicated in the statement of contribution.
2. Due acknowledgement has been made in the text to all other material used.
3. The thesis is less than 100,000 words in length, exclusive of tables, figures, references and appendices.

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Statement of contribution

The completion of this thesis would not have been possible without the following contributions:

All members from Papua New Guinea, Malawi and Australia involved in the collection, processing and shipment of plasma samples and the clinical datasets.

Ms. Wina Hasang helped culture the Var19 infected erythrocyte line, select it for EPCR binding using rabbit anti-var19 antibody (generated by Professor Joseph Smith and provided by Professor James Beeson) and carry out the opsonic phagocytosis assay using the Var19 line for chapter 5.

Dr. Amy Chung and Mr. Timon Damelang analysed the data using MATLAB with machine learning for chapter 6.

All other co-authors of the publication presented in chapter 4 contributed to the work presented in the publication as follows:

Experimental procedures: Janavi S. Rambhatla, Louise Turner (coupling of proteins to beads and provision of experimental details). The experiments were carried out in Dr. Thomas Lavstsen's laboratory at the University of Copenhagen, Denmark.

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Publications and awards

Publications

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- Tonkin-Hill GQ, Trianty L, Noviyanti R, Nguyen HH, Sebayang BF, Lampah DA, Marfurt J, Cobbold SA, **Rambhatla JS**, McConville MJ, Rogerson SJ. The Plasmodium falciparum transcriptome in severe malaria reveals altered expression of genes involved in important processes including surface antigen–encoding var genes. PLoS biology. 2018 Mar 12;16(3):e2004328.

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ABE	Assay buffer E
ACT	Artemisinin-based combination therapy
AGRF	Australian genome research facility
AMA-1	Apical membrane antigen-1
ATS	Acidic terminal segment
BSA	Bovine serum ambumin
BCS	Blantyre coma score
◦C	Celsius
CC	Community controls
CD36	Cluster of differentiation 36
CIDR	Cysteine-rich interdomain regions
CM	Cerebral malaria
Conv.	Convalescent
CO_2	Carbon dioxide
CR1	Complement receptor 1
CSA	Chondroitin sulphate A
CSP	Circumsporozoite protein
DBL	Duffy binding-like
DC	Domain cassettes
DHE	Dihydroethidium bromide
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid

EBA-175	Erythrocyte binding antigen
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
EPCR	Endothelial protein C receptor
FBS	Foetal bovine serum
FU	Follow-up
gDNA	Genomic Deoxyribonucleic acid
GLURP	Glutamate-rich protein
HEPES	4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid
HIHS	Heat-inactivated human serum
HREC	Human research ethics committees
HRP2	Histidine-rich protein 2
ICAM-1	Intracellular adhesion molecule-1
IE	Infected erythrocyte
IgG	Immunoglobulin G
IPTp	Intermittent preventive treatment in pregnancy
IQR	Interquartile range
IRS	Indoor residual spraying
ITNs	Insecticide-treated nets
kDa	Kilo-daltons
L	Litre
LB	Luria Bertini
LLIN	Long-lasting insecticide treated bednets
LOOCV	Leave one out cross validation
Melb. Neg.	Melbourne negative controls

MFI	Median fluorescence intensity
mL	Milli litre
mm	millimetre
mM	millimolar
MQ H_2O	MilliQ water
mRNA	Messenger ribonucleic acid
MSE	Mean square error
MSP	Merozoite surface protein
NaCl	Sodium chloride
NTS	N-terminal segment
OD	Optical density
O/N	Overnight
PAM	Pregnancy associated malaria
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PCA	Principal component analysis
PECAM-1	Platelet/endothelial cell adhesion molecule
PEXEL	Plasmodium export element
PFA	paraformaldehyde
P. falciparum, Pf	Plasmodium falciparum
PfEMP1	Plasmodium falciparum erythrocyte membrane protein 1
PfRH5	P. falciparum reticulocyte binding protein homolog 5
Pfs	Pre fertilization antigens
pLDH	Plasmodial lactate dehydrogenase

PLSDA	Partial least squares discriminant analysis
pmol	Picomole
PNG	Papua New Guinea/ Papua New Guinean
PPS	Pooled positive plasma
PTEX	Plasmodium translocon of exported protein
RBC	Red blood cells
RDT	Recombinant DNA technology
RE	Restriction enzyme
RIFINS	Repetitive interspersed family
RNA	Ribonucleic acid
RNAseq	RNA sequencing
rpm	Rotations per minute
RPMI	Roswell park memorial institute
RT	Room temperature
RU	Relative units
SDS	Sodium dodecyl sulphate
SDS-PAGE	SDS polyacrylamide gel electrophoresis
SERA-5	Serine-rich antigen family
SM	Severe malaria
SP	Sulfadoxine-pyrimethamine
STEVORS	Sub-telomeric variant open reading frame
SURFINS	Surface-associated interspersed gene family
TM	Transmembrane
Tm	Melting temperature

TBV	Transmission blocking vaccines
TRI	Transmission reducing immunity
µg	microgram
µl	Microlitre
UM	Uncomplicated Malaria
Ups	Upstream sequence
VCAM-1	Vascular cell adhesion molecule 1
VSA	Variant surface antigens
WGCFS	Wheat germ cell free protein synthesis systems
w/v	Weight/volume
WHO	World Health Organisation

1 Chapter 1: Literature review

1.1 Introduction

The term malaria has its origins in Medieval Italian: *mala aria*, which translates as bad air, and was previously known as *ague* or *marsh fever*. Malaria is the world's most important tropical and sub-tropical parasitic disease and is transmitted through the bite of the female *Anopheles* mosquitoes.

1.1.1 Epidemiology

Majority of cases occur in children under 5 years old, with pregnant women also being vulnerable. Malaria is presently endemic in tropical and sub-tropical regions and major incidences occur in sub-Saharan Africa (Figure 1.1). As per the latest World Health Organization report in 2017, 216 million cases of malaria were reported worldwide in 2016, with an estimated 445,000 deaths [1]. Climatic conditions such as rainfall, temperature and humidity, transmission intensity, type of vector and parasite causing the disease, along with fluctuations in these factors, are all contributors of malaria transmission and thus malaria epidemiology [2, 3]. The epidemiology of malaria is dependent on whether the malaria transmission in a particular area is stable or unstable [3]. Malaria transmission has been described briefly in section 1.2 of this chapter.

Malaria epidemiology is best described by a quote from the malariologist Lewis Hackett (1937): "Everything about malaria is so moulded by local conditions that it becomes a thousand epidemiological puzzles. Like chess, it is played with a few pieces but is capable of an infinite variety of situations" [4].

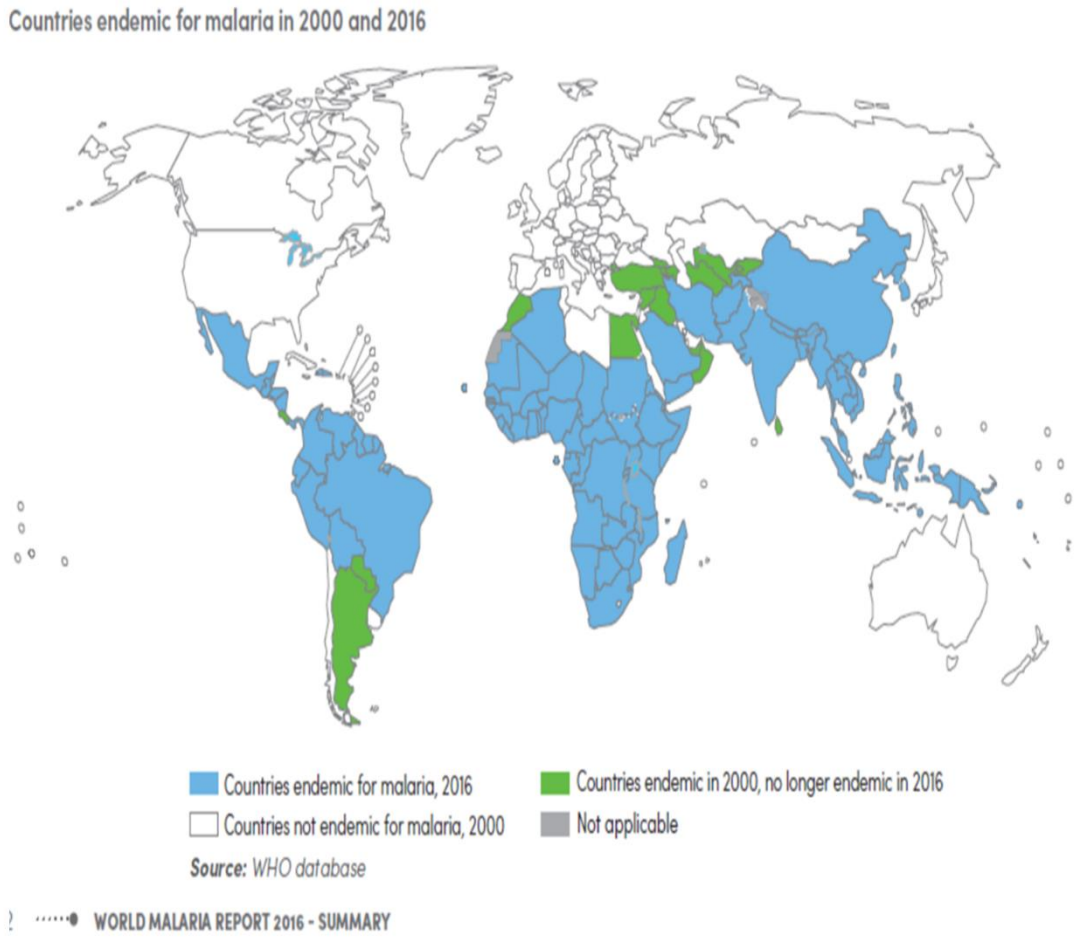

Figure 1.1 Malaria endemic countries in 2016.

This map represents the distribution of malaria in 2016. The countries mapped in blue are endemic for malaria as of 2016 and those in green represent countries which were endemic in 2000, but not as of 2016 [5].

1.1.2 The malaria parasite and its life cycle

Malaria parasites have a complex life cycle. Five species of *Plasmodium*, namely, *P. falciparum*, *P. ovale*, *P. vivax*, *P. malariae* and *P. knowlesi* are capable of infecting humans, all of which have very similar life cycles. *P. vivax* and *P. falciparum* malaria infections are more prevalent than the others. While *P. vivax* infections are more common outside of Africa, especially in the Middle East, Asia and the Western Pacific and can lead to death, it is *P. falciparum* that is responsible for the high malaria mortality [6]. *Plasmodium* is an apicomplexan protozoon that propagates within and destroys host erythrocytes after invasion with the help of its specialised apical complex. Infections last for up to months or years.

Infection begins with the bite of an infected female *Anopheles* mosquito, which injects the sporozoites (infective stage of the malaria parasite), into the human host. After circulating in the blood stream for a short period of time, the sporozoites invade the liver cells, where they develop into exoerythrocytic schizonts over the next 5 to 15 days. After dividing mitotically, 10,000 to 30,000 merozoites which are contained in the exoerythrocytic schizont are released and can now invade the red blood cells. This invasion is dependent on interactions between specific receptors of the erythrocyte membrane and surface ligands of the merozoite. This entire process is completed in about 30 seconds. Within the erythrocyte, the merozoite undergoes erythrocytic schizogony, developing first into the ring stage, then the trophozoites and finally into schizont stages. In order to enhance its chances of survival, the parasite modifies its host cell in several ways. The schizont-containing erythrocyte finally ruptures, releasing the merozoites, which can now invade additional erythrocytes. This process is repeated indefinitely, thus causing the disease (Figure 1.2) [7].

During this asexual, blood-stage development cycle, some of the merozoites develop into female macrogametocytes and male microgametocytes, which are the sexual forms of the parasite. This process is called gametocytogenesis. The timeframe of gametocytogony varies based on the type of *Plasmodium* species infecting the host. For *P. falciparum*, development of mature gametocytes (from stage I to V) requires more than 9 days [8]. The mature gametocytes are then taken up by the mosquito where further differentiation into gametes takes place in the midgut. Thus, in the female *Anopheles* mosquito, the mature macrogametocytes differentiate into macrogametes after exiting the erythrocyte, while the microgametocytes exflagellate to form 8 microgametes. The microgamete fertilizes the macrogamete to form a zygote. The zygote elongates into a motile ookinete within 18 to 24 hours. The ookinete moves across the peritrophic

membrane and epithelial cell of the midgut and forms an oocyst underneath the basement membrane of the midgut epithelium. Within 7 to 15 days post infection, a single oocyst forms more than 10,000 sporozoites, depending on the *Plasmodium* species and the ambient temperature. The motile sporozoites migrate and accumulate in the acinar cells of the salivary glands. When this infected mosquito bites a susceptible vertebrate host, the *Plasmodium* life cycle begins once again [7].

1.1.3 Host cell entry

The erythrocytic merozoites invade other erythrocytes in four general steps: the polar merozoites recognize the erythrocytic membrane and attach loosely to it. They then reorient and form tight junctions between the apical end of the merozoite and release rhoptry-microneme substances with vacuole formation. This is followed by the movement of the tight junction from the apical to the posterior end of the merozoite with the help of an actin-myosin motor powered by the parasite. The merozoite's surface coat is shed with the help of a serine protease (SUB2). As the parasite invaginates on to the host cell, it forms a parasitophorous vacuole to ensure a hospitable environment for its development within the host. Finally, the parasitophorous vacuole membrane and erythrocyte membrane are resealed after the merozoite invasion is completed [9-13] (Figure 1.2).

1.1.4 Protein trafficking

The parasitophorous vacuolar membrane (PVM) of the parasite separates the cytoplasm of the infected erythrocytes (IE) from the vacuole in which the parasite resides. The *Plasmodium* translocon of exported protein (PTEX), which is a parasite protein complex, traffics numerous parasite proteins across the PVM. The PTEX translocon is made up of five different proteins, of which the EXP2 protein forms a pore. The parasite proteins to be transported to the red blood cell (RBC) surface are first unfolded after which they pass through the pore in an energy-dependent manner with the help of a chaperone protein, HSP101 [14]. Majority of the proteins that are to be transported have a *Plasmodium* export element (PEXEL) motif, which is a sequence of five amino acid residues [15]. An enzyme called plasmepsin V causes a cleavage within this motif, thus committing this now mature protein for export to the host cell [16]. The mechanism underlying the transport of a large number of proteins that lack the PEXEL motif is still unknown [17].

Plasmodium falciparum erythrocyte membrane protein 1 (PfEMP1) has an atypical PEXEL motif, which assists in its transport, as cleavage by plasmepsin V does not occur [15]. Maurer's clefts, which are specialized sorting organelles of the parasite, and the surface knobs are involved in the ultimate transport and thus insertion of PfEMP1 in the

host RBC [18] (Figure 1.2). A protein of Maurer's cleft, known as PfSBP1 (skeletal-binding protein 1) is required for PfEMP1 transport to the host cell surface [19, 20].

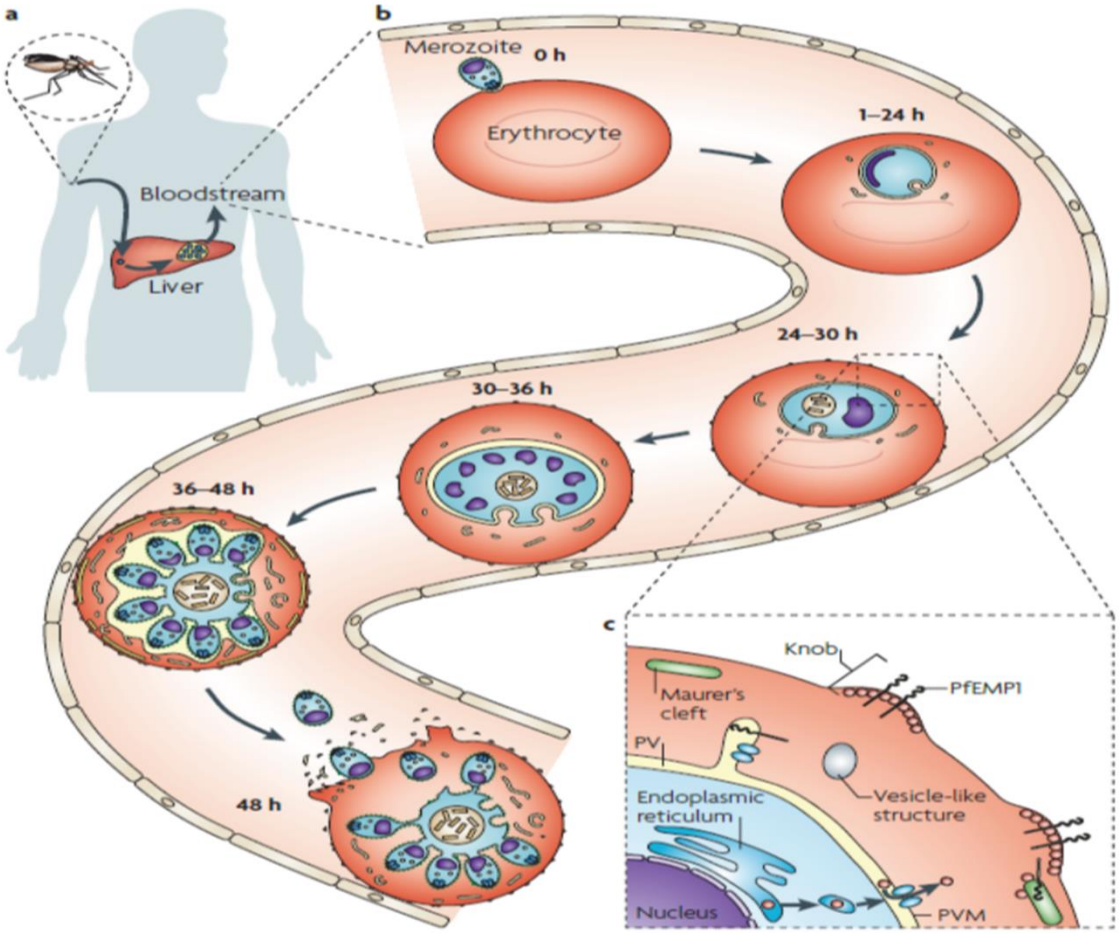

Figure 1.2 Asexual life cycle and the protein trafficking pathway of the parasite.
A: *P. falciparum* has a complex life cycle and requires two hosts: 1) mosquito and 2) human. The life cycle begins with the bite of an infected female *Anopheles* mosquito. B: Within the human host the parasites first take residence in the liver after which they enter the bloodstream. Here, the parasite follows a 48-hour life cycle which comprises of several asexual, blood stages. C: During the asexual stages, various proteins such as the *Plasmodium falciparum* erythrocyte membrane protein 1 (PfEMP1) are exported to the surface of the red blood cell with the help of the Maurer's cleft and surface knobs (adapted from [21]).

1.2 Malaria transmission

Malaria is transmitted by the female *Anopheles* mosquito species infected with the parasite. Transmission intensity is the frequency with which people living in a particular area are bitten by the mosquitoes carrying the *Plasmodium* sporozoites. Parasite prevalence (parasite rate) is often used either as a measure of endemicity or as an indirect measure of transmission intensity and is expressed as a ratio. When infections occur in a community, parasite prevalence is indicative of the presence of parasites in the peripheral blood-stages of infection [3].

1.2.1 Malaria transmission in Papua New Guinea

Over 60% of the population of Papua New Guinea (PNG) lives in areas where malaria transmission is endemic while the remaining 40% live in epidemic-prone areas [22, 23]. Malaria transmission is mainly influenced by the altitude [22], however, malaria endemicity due to small-area variations are also common [24]. Perennial high intensity transmission is prevalent along the coastal and lowland inland regions of the north coast of the main island, especially Madang and East Sepik Province (reviewed in [25]).

The main vector for transmitting malaria in these areas is the *Anopheles punctulatus* species with various other species also contributing to the transmission of the disease [26]. *P. falciparum* and *P. vivax* are the two most frequently infecting species of the parasite and also common causes of severe malaria in children from these areas [27]. Over the last ten years, malaria transmission in PNG has declined due to the distribution of long-lasting insecticide treated bednets (LLIN). As per the 2015 survey, after distribution of LLINs, the weighted average for malaria parasite prevalence in PNG was found to be 12.1%. Parasitaemia was shown to be dependent on age, with children having *P. falciparum* malaria being 5-9 years and children with *P. vivax* malaria having a peak age of 1-4 years [28].

1.2.2 Malaria transmission in Malawi

The entire population of Malawi is at risk of being infected with malaria, which is endemic in more than 95% of the country [29]. Although malaria transmission is high all through the year, intensity varies as per the season [30]. The primary vector causing malaria in these regions is *Anopheles funestus*. The low-lying areas with high humidity and temperatures, such as Shire river valley or along the lakeshore or central plains are at highest risk of infection [31]. *P. falciparum* infections account for almost 98% of the malaria cases as well as severe forms of the disease and lethality [29]. Indoor residual spraying (IRS) along with LLINs are the main methods employed to reduce transmission

in these areas [29]. Children under five years of age are mainly affected by malaria. 10 % of these children are infants below six months who are hospitalized with the disease [32].

1.3 Malaria diagnostics

Light microscopy was one of the earliest methods used for malaria diagnosis and is widely used till date. Giemsa-stained thick and thin smears of blood films are used for this purpose. Rapid diagnostic tests (RDT) are the next most popular methods of detection, particularly in areas where microscopy is not readily available. These are immunochromatography based tests, with soluble histidine-rich protein 2 (HRP2) and plasmodial lactate dehydrogenase (pLDH) being the major malarial antigen targets (reviewed in [33]). Molecular methods using nucleic acid detection tests are also used for malaria detection. Different forms of polymerase chain reaction (PCR) such as nested PCR [34], single-round, multiplex PCR [35] and real-time (qPCR) [36] have been employed for this purpose. To overcome the difficulties in using the traditional PCR machines and real-time PCR, loop-mediated isothermal amplification (LAMP) of parasite DNA was developed [37, 38]. More recently, volatile organic compounds produced by *P. falciparum*, such as plant-like terpenes and terpene derivatives were hypothesized to be present in the breath of malaria patients which in turn could be used to detect presence of malaria [39]. This hypothesis was successfully tested in field samples from Malawi, where detection was >80% in children with fever, thus giving rise to the possibility of developing a breath-based diagnostic test [40]. However, this approach requires more work as the 'breath-print' identified in Malawi was different from that generated in other studies [41].

1.4 Clinical symptoms

Uncomplicated malaria

Uncomplicated malaria presents itself through various symptoms such as fever, chills, headaches, sweats, nausea and vomiting, body aches and general malaise. The symptoms are caused due to the asexual or blood stages of the parasites and are observed around 10-15 days post-infection. If left untreated, uncomplicated malaria can progress into severe malaria.

Severe malaria

The most serious manifestations of malaria are caused by *P. falciparum*, which is also the cause of mortality in majority of the cases [1]. The following paragraph discusses severe malaria in African and Asian regions (although studies from Asia are limited). Infection with this parasite mostly leads to uncomplicated malaria, with <1% of cases developing a more severe form of the disease (reviewed in [42]). However, the number of deaths

caused due to severe malaria is still high. In a study from Asia, mortality rates from severe malaria were 6.1% in children <10 years and 35.6% in adults over 50 years of age [43]. As per the WHO report on severe malaria, the most frequent manifestations of severe malaria in children are cerebral malaria, severe anaemia, metabolic acidosis and respiratory distress, while adults tend to show multiple organ failure as well [44] (Table 1.1). Cerebral malaria is one of the causes of malarial deaths and can lead to long term neurological deficits in some cases as reported in [45], which mainly looked at studies from African children. In the same Asian study described above [43], while severe anaemia was more common in children under 10 years of age, jaundice, acute respiratory distress syndrome, renal failure and pulmonary oedema were causes of severe malaria in older children and adults. Studies from Asia and Africa reported that children and adults showed presence of respiratory distress, which can also be used as a marker of metabolic acidosis [43, 46]. Cerebral malaria and metabolic acidosis or respiratory distress have been shown to be the major contributors of malaria mortality in Asian adults and children [43] and African children [46].

Thus, severe malaria occurs due to a combination of multiple factors, which differ based on age of the individual [47]. Adhesion and sequestration of the parasite IE to various host receptors is a major contributor to this phenomenon (reviewed in [42]).

Table 1.1 WHO defined severe malaria manifestations in children and adults.

Prognostic value (+ to +++)			Frequency (+ to +++)	
Children	**Adults**	**Clinical manifestations**	**Children**	**Adults**
+++	+++	Impaired consciousness	+++	++
+++	+++	Respiratory distress (acidotic breathing)	+++	++
+	++	Multiple convulsions	+++	+
+	+	Prostration	+++	+++
+++	+++	Shock	++	+
+++	+++	Pulmonary oedema (radiological)	+/-*	+
+++	++	Abnormal bleeding	+/-*	+
++	+	Jaundice	+	+++
		Laboratory indices		
+	+	Severe anaemia	+++	+
+++	+++	Hypoglycaemia	+++	++
+++	+++	Acidosis	+++	++
+++	+++	Hyperlactataemia	+++	++
++	++	Renal impairment†	+	+++
+/-	++	Hyperparasitaemia	++	+

The different symptoms associated with severe malaria as defined by WHO in children and adults separately has been described (adapted from [44]).

*Infrequent.

† Acute kidney injury

1.5 Cytoadherence and sequestration

P. falciparum avoids destruction by the spleen through various mechanisms. Cytoadherence, which is the adherence of IE to the vascular endothelium, is one such mechanism. This sequestration in postcapillary venules of various organs leads to organ specific damage and lethal syndromes and can result in severe manifestations of the disease, such as cerebral malaria [48] or placental malaria [49]. Sequestration is predominantly observed during the latter half of the intra-erythrocytic asexual growth phase of the parasite, that is, mature parasites (trophozoites or schizonts) [50].

Knob structures expressed on the IE are an important factor facilitating the sequestration of *P. falciparum* [51]. Knob-associated histidine-rich protein (KAHRP) or Histidine-rich protein 1 (HRP1) is one of the major structural components of the knobs [52]. Reduced cytoadhesion is observed in parasites having disrupted KAHRP genes which result in improper knob formation [53]. However, importance of knobs in sequestration is not conclusive as studies in *P. malariae* have shown that these parasites do not sequester despite the presence of knobs [54], while *P. chabaudi* parasites sequester even in the absence of knobs [55].

These knobs present PfEMP1 on the IE surface. PfEMP1 is a major virulence factor of the parasite and is responsible for the cytoadhesion of the IE to the host microvascular endothelial cells [56-58] through interactions with various host molecules such as intercellular adhesion molecule 1 (ICAM-1) [59], cluster of differentiation 36 (CD36) [60], complement receptor 1 (CR1) [61], heparin sulphate (HS) [62], endothelial protein C receptor (EPCR) [63] and gC1qR [64]. Chondroitin sulphate A (CSA) is another receptor to which the IE adheres [65], particularly the IE from the placenta [66].

Some of the other proteins associated with sequestration are histidine-rich protein II [67], PfEMP2 or mature erythrocyte surface antigen [68], PfEMP3, ring-infected erythrocyte membrane surface antigen (Pf155/RESA) [69], sequestrin and rosettins [70]. Thrombospondin (TSP) [71], platelet/endothelial cell adhesion molecule (PECAM/CD31) [72] and vascular cell adhesion molecule 1 (VCAM-1) [73] are some other receptors implicated in cytoadherence and sequestration.

Rosetting, that is, spontaneous binding of infected erythrocytes to uninfected erythrocytes is another such mechanism through which the parasite cytoadheres [74]. Rosetting is mediated by three major variant surface antigens: PfEMP1, repetitive interspersed family (RIFIN) and surface-associated interspersed gene family (SURFIN) (reviewed in [75]). Clumping of IE or autoagglutination is another adhesive phenotype attributed to IE [76]

which was later shown to be mediated by platelets (probably through platelet glycoprotein CD36) and hence called platelet-mediated clumping [77].

Thus, the parasite shows diverse binding properties, which in turn helps it sequester within tissue vascular beds and maintain the ability to vary the antigenic properties of PfEMP1 (Figure 1.3). Switches in PfEMP1 expression [76] leads to changes in the parasite's sequestration properties and virulence, thus allowing it to escape the host's immune system. Thus, it can be said that two opposing selection pressures act on an infecting parasite during its life cycle: optimal cytoadherence (functional selection) and avoiding immune recognition by expressing variant surface antigens to which the person lacks pre-existing antibodies [78].

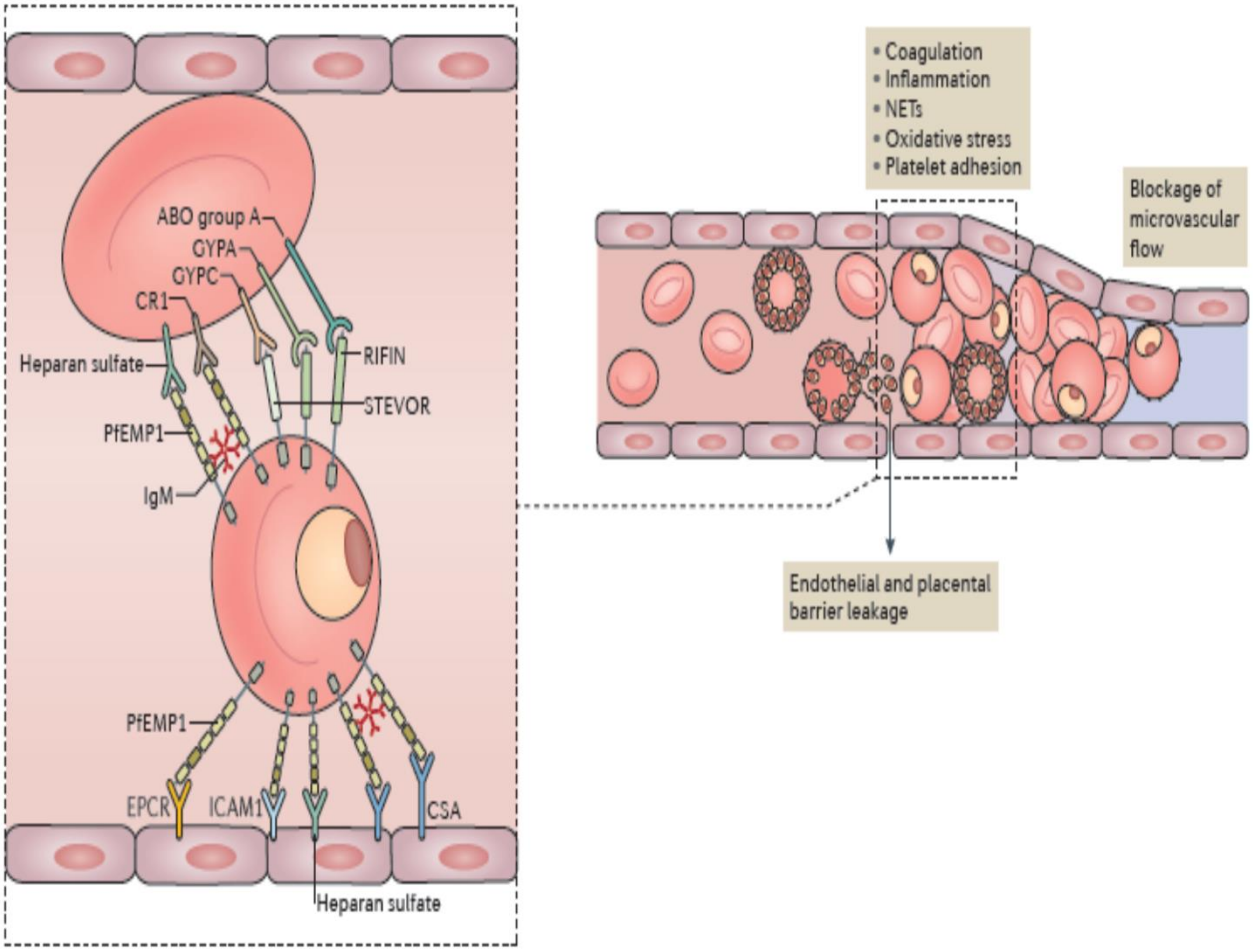

Figure 1.3 Cytoadherence and sequestration of the infected erythrocyte (IE).

The IE can bind to various endothelial host receptors (although not at the same time) such as heparan sulfate, blood group antigens, endothelial protein C receptor (EPCR) and intercellular adhesion molecule 1 (ICAM-1) through the variant surface antigens (VSA) expressed by the parasite on the surface of the red blood cell (VSAs can have functions other than IE adhesion as well). The IE can also bind to chondroitin sulphate A (CSA). The VSAs include Plasmodium falciparum erythrocyte membrane protein 1 (PfEMP1), repetitive interspersed family (RIFIN) and sub-telomeric variable open reading frame (STEVOR). This cytoadherence and sequestration can lead to tissue specific damage and severe malaria through blockage of microvascular flow, disruption of endothelial and placental barriers, coagulation, oxidative stress and inflammation (adapted from [79]).

1.6 Variant surface antigens (VSA) on the infected erythrocyte surface

VSAs are proteins expressed by *P. falciparum* which allows them to adhere to the host vasculature thus increasing their virulence. It is important to note that VSAs can have functions other than IE adhesion. VSAs are variable and diverse due to clonal antigenic variation. Some of the well-known VSAs are PfEMP1 [58], RIFIN [80, 81], sub-telomeric variable open reading frame (STEVOR) [80, 82] and SURFIN [83]. *P. falciparum* Maurer's cleft two transmembrane (PfMC-2TM) [84] and parasite -modified erythrocyte band 3 have also been proposed as VSAs (reviewed in [54]). PfEMP1 is the most extensively studied VSA, and parasites usually do not express PfEMP1 alone [85], but also RIFINS [86, 87] and STEVORS [88] (figure 1.3).

1.6.1 Repetitive interspersed family (RIFIN)

RIFINs are encoded by 150-200 *rif* genes per parasite genome. These are clonally variant, 30-45 kDa proteins, belong to one of the largest multigene families [80, 81] and appear on the surface of the IE at the same time as PfEMP1 [89]. Chromosomal location of these *rif* genes is predominantly sub-telomeric [80, 90]. A single parasite can transcribe multiple *rif* genes and thus different variants of RIFIN proteins can be expressed on the surface of the IE simultaneously [86]. *Rif* genes are transcribed around 12 hours post-invasion [89] and the proteins are transported to the IE surface with the help of the PEXEL or host-targeting (HT) signal present on the RIFIN sequences [15, 91]. Other than the asexual stages of the parasites, RIFINS are also expressed by sporozoites, merozoites and gametocytes [92, 93]. RIFINs can be classified into two groups: A-type, which are exported to the IE surface via the Maurer's cleft and B-type, which are not exported and remain within the parasite [92]. The biological function of RIFIN is not completely defined, but they have been shown to confer antigenic variation to the parasite [94]. Studies have also shown RIFINs to elicit immune responses which are stable over time and capable of rapidly clearing the circulating parasites [94, 95]. A-RIFINs were recently shown to mediate rosetting of IE belonging to blood group A and O [87].

1.6.2 Sub-telomeric variable open reading frame (STEVOR)

STEVOR proteins are 30-40 kDa proteins encoded by the third largest multigene family of *P. falciparum*: *stevor* [80]. These genes also are sub-telomerically located on the chromosome [80, 90]. The proteins are coded by 30-40 *stevor* genes [80], which are transcribed ~ 28 hours post-infection [82, 89]. A single parasite transcribes multiple *stevor* genes during its late trophozoite and schizont stages. The proteins are initially found in the cytosol and with time they were found to localize in the Maurer's cleft [82, 96]. Later

studies have demonstrated that these proteins are present on the surface of the IE during the schizont stage of the parasite's life cycle and are clonally variant, thus contributing to the antigenic variation of the parasite [88]. As with RIFINS, STEVOR proteins also contain the PEXEL motif [15, 91]. STEVOR proteins were also shown to be expressed by other stages of the parasites: merozoites [97] and gametocytes [82]. Some of the proposed functions for these proteins are increasing the rigidity and stiffness of the IE, which in turn may boost the PfEMP1-mediated sequestration of IE [98], and involvement in parasite invasion during the merozoite stages [99, 100]. STEVORS were also shown to mediate rosetting of schizonts in a PfEMP1-independent manner [100].

1.6.3 Surface-associated interspersed gene family (SURFIN)

There have been few studies explaining the role of SURFINs during a malaria infection. The *surf* multigene family comprising of 10 genes, encode the SURFIN proteins, which are ~280-300 kDa in size. These are expressed by merozoites and on the IE surface as well. Along with RIFINs and PfEMP1, SURFINs are also transported to the Maurer's cleft and eventually to the RBC surface. During the parasite's development, different *surf* genes are transcribed during different stages of the parasite [83]. $SURFIN_{4.2}$ variant may be involved in merozoite invasion of erythrocytes [83] and rosette formation [101], however, further studies are required to corroborate these findings.

1.6.4 *Var* genes

Var genes were first reported in 1995 as the gene family that encodes the PfEMP1 protein. The molecular size of these genes varies from 6-15 kb; they are extremely divergent in sequence and are capable of encoding 200-500 kDa sized proteins. Exon I and Exon II of the *var* gene code for the PfEMP1 protein. While Exon I codes for the variable, extracellular region and the transmembrane region of the protein, Exon II codes for the relatively conserved intracellular region of the protein (acidic terminal segment or ATS) [102-104].

Mature (IIB-V) and immature (I-II) stage gametocytes also transcribe *var* genes and express PfEMP1 variants [105]. Using in vitro transcription studies, gametocytes were shown to transcribe group C *var* genes. Further, it was demonstrated that the transcriptional profile of *var* genes in gametocyte-IEs was not linked to the *var* expression seen in asexual parasite stages [106]. The focus of this thesis is the asexual stages of the parasite, and hence *var* genes and PfEMP1s expressed by these stages are mainly discussed.

P. falciparum has a limited antigenic repertoire. Therefore, control over its variant expression is an important part of its pathogenesis. The *var* gene switching pattern is highly structured and non-random, with an initial dominant transcript switching to a new dominant transcript or back to the original transcript via a series of switch intermediates [107].

Based on the semi-conserved upstream promotor region of the gene encoding PfEMP1 (Ups A, Ups B, Ups C, Ups D and Ups E), the chromosomal location and the direction of transcription, the PfEMP1s can be classified into group A, B, B/A, B/C, C or E. Thus, group A and group B *var* genes are telomeric in location while group C genes are located near the centromeres. While the transcriptional direction for group A genes (Ups A) is towards the telomere, the direction of transcription for group B genes (Ups B) was centromeric [108-110] (Figure 1.4). PfEMP1 coded by Ups D was shown to be a pseudogene with high sequence similarity to *var1* sub-family of *var* genes [108, 111, 112]. *Var1* is mostly found as a truncated gene with its particular functional property largely unknown [110]. Group E PfEMP1 encoded by Ups E, belong to the *var2* sub-family which is associated with placental malaria through CSA binding [108, 113].

This grouping of *var* genes can be linked to exchange of genetic information, with gene recombination occurring more commonly within the groups as compared to between the *var* groups, thus allowing the parasites to overcome the varying conditions of transmission and the immune responses mounted by the host. It has been hypothesized that the grouping is maintained by inhibition of recombination which is facilitated by the different chromosomal location of the genes and the direction of transcription [108].

1.7 Plasmodium falciparum erythrocyte membrane protein 1 (PfEMP1)

PfEMP1 are clonally variant, multi-domain surface antigens, encoded by about 60 *var* genes per parasite genome, expressed in a mutually exclusive fashion, which are then exported to the IE surface where they are exposed to host antibodies [90, 114, 115]. Whether expression of PfEMP1 is mutually exclusive or not has been a point of interest for several years. Using non-immune human volunteers it was found that during the early phase of the blood stage infection, in a population of parasites, at the same time, many different PfEMP1s can be expressed [116]. In another study, using selected 3D7 parasites, two particular *var* genes and therefore two PfEMP1s were expressed at the same time on one IE surface which in turn facilitated binding of the IE to two receptors [117]. A previous study showed that while during the ring stages several *var* gene variants are expressed, upon maturation into trophozoites expression of *var* genes is more controlled. And when selected for a particular binding phenotype, only one

predominant *var* gene and thus PfEMP1 is expressed by the parasite [118]. Similar results were also found for a rosetting parasite IE line [119]. *P. falciparum* infection virulence is characterized by the type of PfEMP1 being expressed on the surface of IE which in turn helps anchor them to the vascular lining [120].

1.7.1 Structure of PfEMP1

The sequence of each *var* gene is unique, but in general each PfEMP1 variant can be said to be composed of (from N- to C-terminal), an N-terminal segment (NTS), duffy binding-like domains (DBL), cysteine-rich inter-domain region (CIDR), one transmembrane region (TM) and the ATS (Figure 1.4). The DBL domains are classified into six major classes, viz, DBL α, β, γ, δ, ε and ζ, which are further characterized by ten semi-conserved homology blocks, HBa-j; while CIDR domains are divided into five classes: α, β, γ, δ and pam [110, 121]. Based on protein architecture, PfEMP1 proteins can be further classified into two groups: a small protein containing four extra-cellular binding regions (DBL-CIDR-DBL-CIDR) or a large protein which has a more complex domain composition [122].

1.7.1.1 Domain cassettes

Domain cassettes (DC) can be defined as sets of PfEMP1 domains that usually occur together (Figure 1.4). Previous studies have identified a set of 23 conserved domain cassettes for PfEMP1, of which *var1*(DC1), *var2csa* (DC2) and *var3* (DC3) are most prominently found in all *P. falciparum* genomes [110]. In some of the *var* genes encoding domain cassettes, a particular semi-conserved sequence present in one part of the gene is predictive of another semi-conserved sequence present in the regions of the gene encoding other domains [120].

1.7.2 Domains, domain cassettes and their binding phenotype

In recent years, DBL and CIDR domains, as well as various domain cassettes of PfEMP1 have been well characterized. These domains, along with their binding phenotypes are described in the following paragraphs. An important point to remember is that the domains found in DCs have also been found outside the DCs and are not always restricted to or diagnostic of a DC. The following paragraphs summarise few of the studies, where certain domains have been described within a domain cassette.

DC8 genes (group B/A) are characterized by UpsB promoter (group B *var* gene) and have arisen due to group A and group B *var* gene recombination, occurring in the DBLα encoding part of the gene, thus sometimes showing group A-like gene characteristics.

DC8 contains DBLα2-CIDRα1.1-DBLβ12-DBLγ4/6 domains [120]. The CIDRα1.1 domain in this DC is responsible for its binding to EPCR [63], while DBLβ12 mediates gC1qR binding [123] (Figure 1.4). Even though domain architecture is conserved across the parasite genomes, the DC8 domains only share between 45%-63% sequence similarity at the amino acid level and are not completely identical [120]. DC8 sequences show antibody-mediated diversifying selection given the fact that they show considerable amino acid polymorphism, as shown for the variable regions of *var2csa* in one study [120, 124].

DC13 belongs to group A *var* genes, includes DBLα1.7- CIDRα1.4 [120] and binds to EPCR through the CIDRα1 domain [63]. This head structure, followed by a DBLβ1 or β3, whichever comes first, binds to ICAM-1, thus facilitating dual binding of DC13 to ICAM-1 and EPCR [125]. However, a recent study showed that under physiological flow conditions, the IE-EPCR interaction mediated by DC8 and DC13 PfEMP1 may be different from that observed with recombinant proteins [126].

The group A *var* genes are further divided in two, based on the type of the first CIDR domain being expressed: CIDRα1 group, which includes CIDRα1.4 to α1.7 and a second group which includes CIDR β, γ and δ [120]. Although CIDRβ/γ/δ domains have not been directly linked to a phenotype, there are a few studies showing that PfEMP1s containing DBLα1- CIDRβ/γ/δ domains may be associated with rosetting [61, 127]. While CIDRα1.1 and/or CIDRα1.4-1.8 PfEMP1 domains bind EPCR [128], CIDRα2-6 domains were shown to mediate CD36 binding [129, 130]. Group A CIDRα domains that do not bind EPCR are CIDRα1.2/1.3 (DC1). Group B CIDRα domains, VAR2CSA or VAR3 also do not show EPCR binding [63].

Early studies found that only those PfEMP1 proteins encoded by group B and group C *var* genes having the DBLβ-C2 domain (from IT4 parasite IE line) (and not group A PfEMP1) were able to bind to ICAM-1 [131]. However, later studies showed that there were some group A PfEMP1 (having the DBLβ-C2 domain (from 3D7 parasite IE line)) which showed strong and specific binding to ICAM-1 [132].

DC4 contains DBLα1.1/1.4-CIDRα1.6-DBLβ3 domains occurring together and binds to ICAM-1 though the C-terminal DBLβ3 domain [133]. This DBLβ-ICAM-1 association in DC4 and DC13 PfEMP1 head structures was also validated in a recent study from PNG [134].

A recent study by Lennartz and group [135], showed that DBLβ domains belonging to group A or B/A *var* genes contain a highly conserved sequence motif (glycine and proline-rich) which facilitates its binding to ICAM-1 and PfEMP1s lacking this sequence motif

failed to bind ICAM-1. DBLβ belonging to groups B or C *var* genes and binding to ICAM-1 did not contain this sequence motif. In addition, they also found that all the PfEMP1s containing this DBLβ domain with the conserved sequence, also had a CIDRα1 domain, which facilitated dual binding of this PfEMP1 to ICAM-1 and EPCR. They found that of all the EPCR-binding PfEMP1s, 14% had the group A ICAM-1-binding, conserved DBLβ motif. The ICAM-1 binding PfEMP1, belonging group B and C were often seen in conjunction with CIDRα domains binding CD36 [129-131, 135, 136].

Thus, the DBLβ and ICAM-1 association can be found in DC4, DC13 as well as other PfEMP1s not associated with any DC and can belong to either group A, B or C *var* types.

DC5 on the other hand binds to PECAM1 and consists of the domains: DBLγ12-DBLδ5-DBLβ3/4-DBLβ7/9 [137]. DC6 comprises of C-terminal PfEMP1 domains: DBLγ14-DBLζ5-DBLε4, which usually occur with either of the four N-terminal head structure domains associated with rosetting, binding to EPCR (group A or DC8) or CD36 [138]. The binding characteristics of DC6 are as yet unknown. In some studies, DBLζ and DBLε domains have been shown to bind to serum factors IgM and α2-macroglobulin which in turn leads to cross-linking of multiple PfEMP1s. This has been hypothesized to increase the binding affinity of the PfEMP1 N-terminal region [139, 140]. DBLζ/ε domains binding to natural IgM antibodies have also been associated with IgM+ rosetting [127]. DC11 contains CIDRβ4-DBLγ7-DBLε11-DBLζ2-DBLε11-DBLε3 [123, 141]. Some of the other DCs that have been characterized are DC16 containing DBLα1.5/6-CIDRδ and DC15 which contains DBLα1.2- CIDRα1.5 domains [120] (Table 1.2).

1.7.3 PfEMP1 and rosetting

PfEMP1 mediated rosetting occurs through binding to CR1 [61], heparan sulfate [62] and blood group A antigens expressed on the surface of RBCs [142]. NTS-DBL1α domain of PfEMP1 has been implicated in rosetting of IE through these receptors [142, 143]. More recently, rosetting parasites infecting the erythrocytes were shown to mediate both cytoadhesion and rosetting at the same time through NTS-DBL1α and DBL2γ domains within a single PfEMP1 protein [144]. Rosetting parasites can also be grouped in two: those binding to the serum factor IgM and those not binding IgM. IgM positive rosettes are caused by parasites expressing an N-terminal DBLα1.5 or 1.8 domain [127] (Table 1.2).

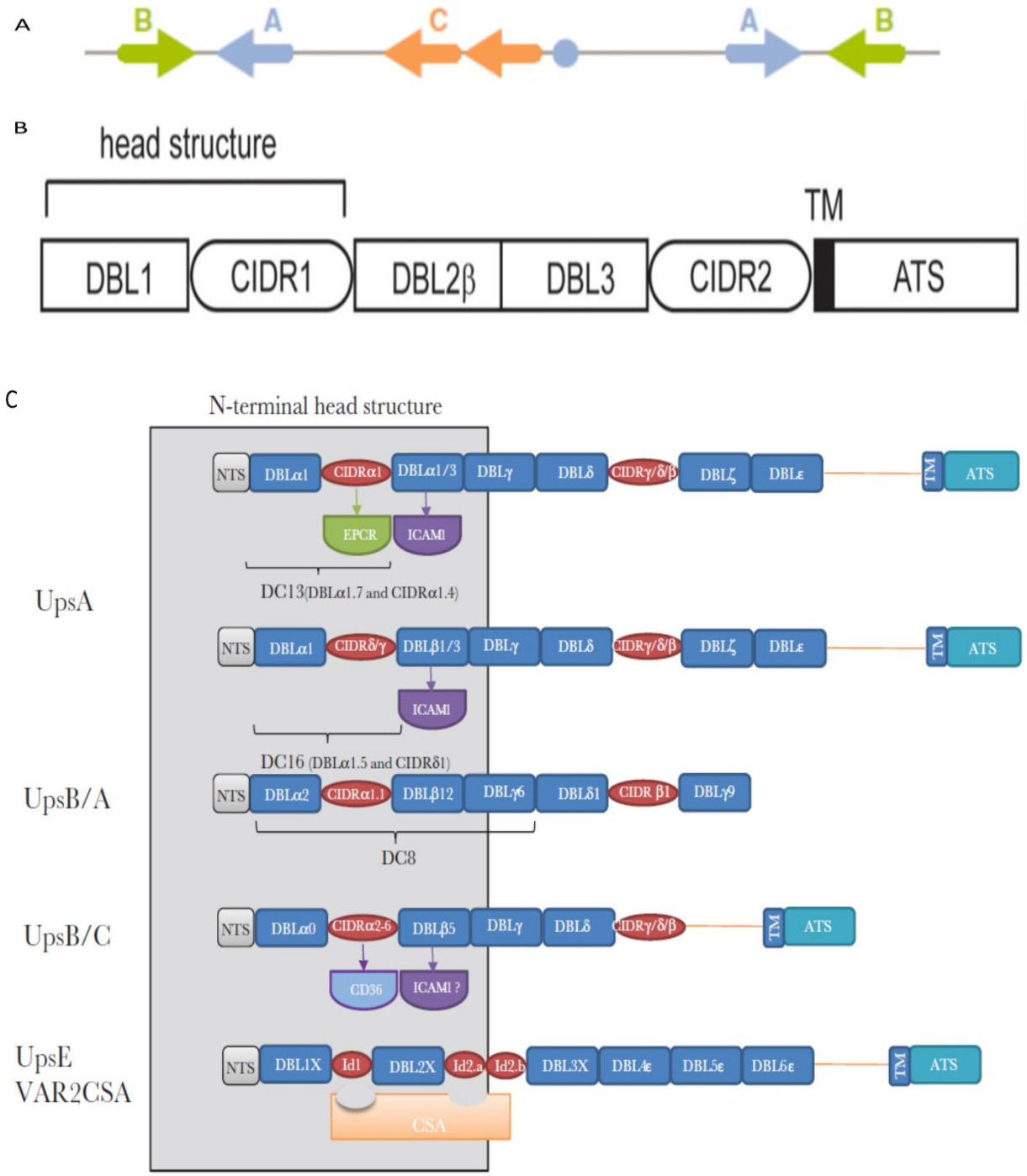

Figure 1.4 *var* genes and PfEMP1 structure.

A: Chromosomal organization of *var* genes based on upstream promotor regions (adapted from [145]) B: Example of the general composition of the multi-domain *Plasmodium falciparum* erythrocyte membrane protein 1 (PfEMP1) (adapted from [146]). C: Examples of some domain cassettes (DC) and other PfEMP1 head structures along with their upstream promotor region. Binding phenotype for some of the domains have also been presented: endothelial protein C receptor (EPCR), intercellular adhesion molecule 1 (ICAM-1) or cluster of differentiation 36 (CD36) (adapted from [147]).

1.8 *var* genes and severe malaria

There have been several studies looking at associations between the three major *var* gene groups: Group A, B and C and disease severity (Figure 1.5). Previous study found that Group B and C *var* genes encode PfEMP1 variants binding to CD36 [136]. Group C *var* genes were associated with asymptomatic malaria in PNG [148, 149], while group A PfEMP1 variants that do not bind to CD36 were associated with severe clinical disease, including cerebral malaria (impaired consciousness) [150]. Expression of group B *var* genes (as well as group A and those *var* genes with a complex head structure) was shown to be associated with clinical malaria in PNG [148, 149]. Similarly, group B (and A) *var* gene transcripts were higher in severe (as well as clinical) malaria in Africa [151], while group B *var* genes were also associated with cerebral malaria (compared to mild or severe malarial anaemia cases) in Africa [152].

In Papua New Guinea, a high proportion of group A *var* sequences were found to be associated with symptomatic malaria, while severe malaria was associated with a sub-group of *var* genes having a complex head structure. Older children having asymptomatic malaria and high levels of parasitaemia expressed a subset of group C *var* genes [148]. Group B *var* gene transcription was upregulated in Papua New Guinean children with clinical severe and mild malaria [149] (Figure 1.5).

Very few studies have found associations between the entire PfEMP1 protein with severe malaria. VAR2CSA is the only exception, which has been implicated in malaria in pregnancy and widely studied. However, there have been several studies linking specific domains or DCs of PfEMP1 with severe malaria or severe malaria syndromes and development of immunity against them [141].

1.8.1 DBL domains and severe malaria

Parasites expressing PfEMP1 with DBLα sequences (characteristic for group A PfEMP1) are associated with severe malaria infections [120, 153-155].

Recently, PfEMP1 containing the group A, DBLβ-specific, ICAM-1 binding motif and CIDRα1, which bind dually to ICAM-1 and EPCR respectively, were shown to be associated with cerebral malaria in children. However, PfEMP1s containing only the EPCR binding CIDRα1 domain were associated with severe malaria only and not any specific severe malaria syndrome [135]. There have been some previous studies linking the adhesion of IE to ICAM-1 and cerebral malaria (in children from Kilifi, Kenya) [156], to clinical malaria in non-anaemic patients, as well as cerebral malaria patients (children) in Kilifi, Kenya [157]. However, studies have also shown an inverse correlation between the

IE-ICAM-1 binding and cerebral malaria in Malawian children [158]. Thus, over the years, associations between DBL domains and severe malaria have been confirmed and have been summarized in table 1.2.

1.8.2 CIDRα1 domains and severe malaria

Studies have shown that children with severe malaria have parasites that express those PfEMP1's which have DC8 (observed in majority of the cases, regardless of symptomology) or DC13 and that these PfEMP1 types show similar traits. DC5 transcription may also be high in such cases, although such high levels were rarely observed by themselves [120]. As most parasites have *var* genes that encode DC8 PfEMP1, it is suggested that DC8 PfEMP1 leads to severe malaria due to its cytoadhesive properties in malaria endemic regions [120]. Further investigations revealed CIDRα1 domains from these DCs to be involved in EPCR binding [63]. *Var* gene studies in Tanzanian children with severe malaria showed that the PfEMP1s expressed by the parasites all had a CIDRα1 domain in common highlighting the role of this domain in severe paediatric malaria [159]. Similar results were also found in Indian adults, where DC8-containing PfEMP1s were overexpressed in severe malaria cases [138]. Children with cerebral malaria and brain swelling (on magnetic resonance imaging) showed high transcript levels of DC8 and group A PfEMP1 types, with CIDRDα1.7 domain being predominantly expressed, which inhibited the protective functions of EPCR [160]. CIDRα1.5 was the most abundant EPCR-binding CIDR domain observed in child with cerebral malaria from PNG [161]. Studies in children aged 6 years or less from Benin have shown that the transcript levels for CIDRα1.4 and CIDRα1.5 domain subclasses were higher in parasites causing severe disease as compared to uncomplicated malaria and that these domains may be involved in cytoadhesion [147]. The same study further demonstrated the importance of EPCR and ICAM-1-binding PfEMP1 transcripts in children with cerebral malaria. DC8 transcripts containing the DBLβ12 domain which bind to gC1qR, were higher in Mozambican children with severe anaemia as compared to children with uncomplicated malaria [123]. In summary, these studies suggest that EPCR binding CIDRα1 domain transcripts are upregulated in children and adults with severe malaria. These studies have been summarised in table 1.2.

EPCR is an important receptor on the host endothelium and has cytoprotective, anti-inflammatory and anticoagulative functions (reviewed in [162]). Given that parasites expressing EPCR-binding DC8 and DC13 PfEMP1 cytoadhere to endothelial cells in the brain as well as other regions, these PfEMP1s can be further exploited as targets for

interventions to treat severe malaria (particularly cerebral malaria) and prevent them [163].

1.8.3 Other domain cassettes and severe malaria

DC5 transcript levels were shown to be upregulated in severe malaria cases from Africa [120], Papua, Timika [141] and one cerebral malaria case from PNG [161]. A study from Mozambique showed that DC11 may be associated in the pathophysiology of severe malaria as DC11 transcript levels were higher in children with severe malaria than uncomplicated malaria [123]. Most recently, in a RNAseq based study from Timika, Indonesia [141], DC11 was also shown to be up-regulated in severe malaria. DC16 gene transcripts, which belong to group A *var* genes and lack the CIDRα1 domain, did not show any associations with severe malaria in Tanzanian children [120]. Analyses using machine learning in an Indian adult population predicted that DC6 and DC8-containing *var* genes in conjunction with high parasite biomass resulted in severe disease and hospitalization of patients [138]. Associations between these DCs and severe malaria have been summarized in table 1.2.

1.8.4 Rosetting and severe malaria

Rosetting (that is, binding of infected erythrocyte to uninfected erythrocytes) has been associated with disease severity in several studies from Africa [153, 164-166] and with respiratory distress in one other study [150]. Among parasites infecting PNG children, rosetting isolates showed significantly higher expression of group A *var* genes [149]. Rosetting was shown to be mediated by NTS-DBL1α PfEMP1 domain [142]. A study from PNG however, found no assocations between severe malaria and rosetting rates [167]. In Malawi, parasites from children with either severe or uncomplicated malaria did not form rosettes [158]. IE clumping mediated by platelets has also been implicated in severe malaria in children from Kenya [77] and patients from Thailand [168]. The link between severe malaria and rosetting is conflicting and requires further investigations (Table 1.2).

Thus, numerous studies have looked at associations between *var* transcripts of PfEMP1 domains and severe malaria (as described above) [120, 135, 141]. Most of these associations were studied in individuals from Africa, with fewer studies focusing on individuals from Asia-Pacific. Studies looking at antibody immunity against these PfEMP1 domains have been described in section 1.9.2.4.

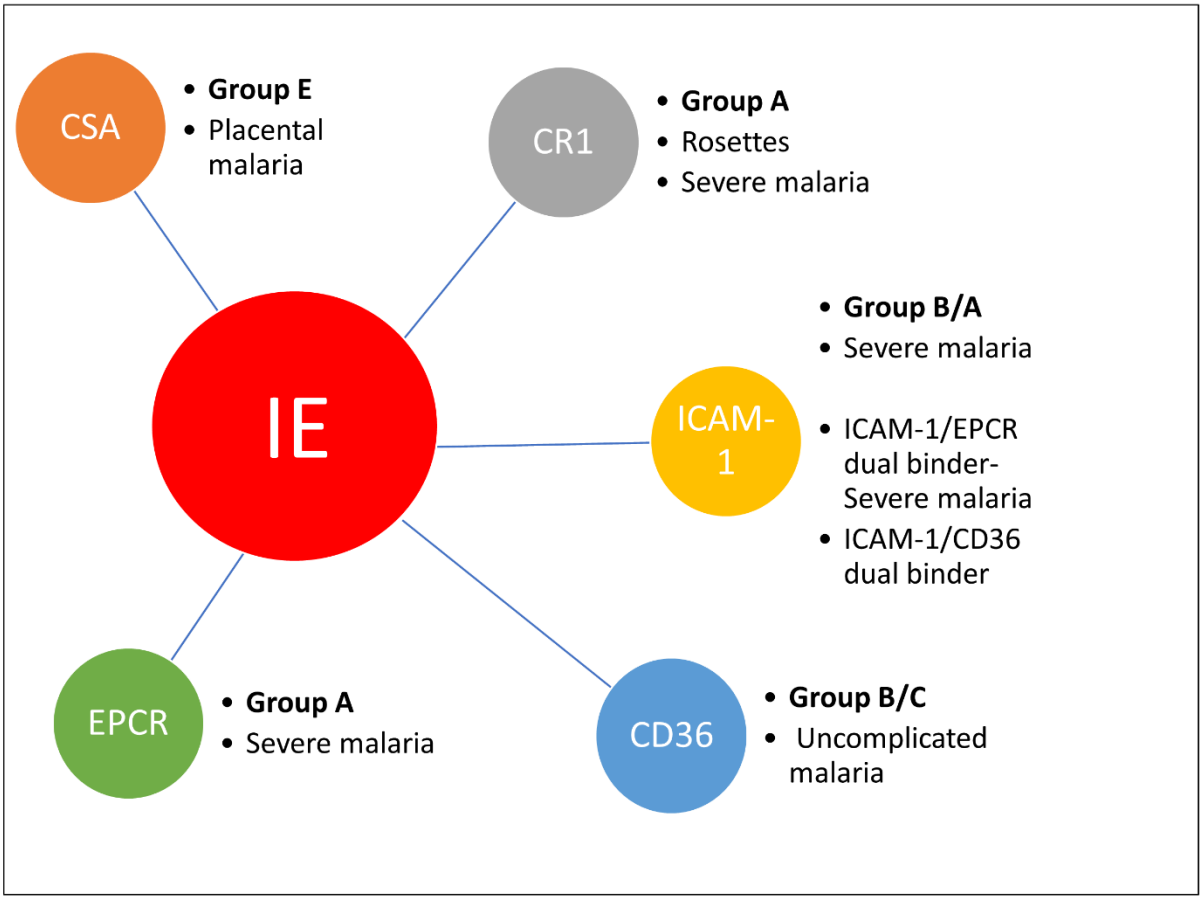

Figure 1.5 Associations of *var* gene groups with malaria severities.

The infected erythrocyte (IE) can bind to various endothelial receptors such as complement receptor 1 (CR1), intercellular adhesion molecule 1 (ICAM-1), cluster of differentiation 36 (CD36) and endothelial protein C receptor (EPCR). It can also bind to chondroitin sulphate A (CSA). The ICAM-1 binders can also dually bind either EPCR or CD36. Binding is through the PfEMP1 protein belonging to different *var* types (Group A, B/A, B/C, E). This grouping is associated with either placental, severe or uncomplicated malaria.

1.9 Immunity to malaria

Understanding the role of immunity against malaria has always been a priority as it is one of the important aspects in development of vaccines against malaria and improving protective outcomes. Numerous reviews over the last decade have highlighted the importance of both cell-mediated and humoral immunity, as well as the interaction between the two in controlling the parasite transmission and protecting from malaria [169-171].

1.9.1 Cell-mediated immunity

Although the targets of cell-mediated immunity are not clearly understood, studies have demonstrated that malarial antigens are presented on major histocompatibility complex (MHC) molecules with the help of antigen presenting cells. This leads to recruitment of CD4+ and CD8+ T-cell responses, which are targeted against the different stages of the parasite's life cycle: liver stage, asexual and sexual stages (reviewed in [171]). Memory T-cells of the liver are important for protection against early stages of the infection [172], and memory CD8+ T-cells function in the presence of CD4+ T-cells [173]. Although RBCs do not express MHC molecules, IE and free merozoites can potentially bind to dendritic cell or macrophage surface receptors. These can then migrate to the spleen, where they encounter antigen specific T-cells [174]. In an early study, αβ+ and γδ+ T-cells that secrete IFN-γ against *P. falciparum* antigens were shown to be involved in cell-mediated immunity and protection from malaria [175]. Later studies revealed that T-cell responses mediate protection through IFN-γ production but not IL4 or IL10 production and do so even in the absence of antibodies [176]. IE-cell-mediated immunity studies revealed that this interaction led to IE being eliminated by activation of natural killer (NK) cells [177]. CIDR1α domains were also shown to play a role in cell-mediated immunity through stimulation of CD4+ T-cells and NK cells, resulting in IFN-γ production, albeit in both malaria exposed and unexposed individuals from endemic regions [178, 179].

Understanding cell-mediated immunity against malaria in co-operation with antibody immunity is crucial, particularly, as T-cell responses were shown to mediate sterile immunity in sporozoite based vaccines [180].

1.9.2 Humoral immunity

Based on the findings from a recent study [181], it was proposed that naturally acquired antibodies against the different blood stage antigens could have different roles in immunity to malaria**.**

This section mainly describes the acquisition of antibody immunity against the different parasite stages and antigen targets expressed by the parasites. Functions of these antibody responses have been described in a later section (section 1.10), titled ‘Role of malarial antibodies’.

1.9.2.1 Antibody immunity to sporozoites

Malaria infection starts when sporozoites are injected into the human host through the bite of the female *Anopheles* mosquito. Once injected, the sporozoites can take several hours before they take residence in the liver [182]. During this time, the sporozoites are susceptible to immune responses mounted by the host [183]. Cicumsporozoite protein is the major surface antigen expressed on the sporozoites and antibodies against CSP can be acquired through malaria exposure [184]. Anti-CSP antibodies have been associated with protection from infection (*P. yeolii*) in animal models [185, 186] and in a human vaccine trail [187]. Development of immunity against sporozoites could potentially reduce hepatocyte infection by sporozoites and thus the following blood stages of infection, thereby conferring protection from clinical malaria [183].

1.9.2.2 Antibody immunity to merozoites

The blood stage of malaria parasite starts with merozoites invading the RBCs. Numerous antigens are presented on the complex surface of the merozoites (including merozoite surface proteins, MSP), which in turn are presented to the hosts immune system (reviewed in [188]). Unlike sporozoites and IE, where CSP [189] and PfEMP1 [190] respectively, are the immunologically dominant proteins expressed on the surface, no single protein is dominantly expressed by the merozoites as immune targets. MSP-1, MSP-2, MSP-3, apical membrane antigen 1 (AMA-1), EBA-175 (erythrocyte binding antigen), GLURP (glutamate-rich protein), *P. falciparum* reticulocyte binding protein homolog 5 (PfRH5) and SERA-5 (serine-rich antigen family) are some of the recent *P. falciparum* merozoite vaccine candidates that have moved towards human vaccine trials (reviewed in [188]). Antibodies targeting these different antigens have been associated with protective immunity. Rhoptery and microneme proteins were found to be most associated with protective antibody responses [191]. Antibody acquisition against merozoite proteins was further studied in a longitudinal cohort from PNG, where the antibody levels were found to be higher in older children with active parasitaemia [191]. As shown in meta- analyses of merozoite antigens, IgG to MSP-3 and MSP-1_{19} was most associated with a relative reduction in malaria risk [192]. In Kenyan children, antibodies against combinations of major merozoite vaccine candidates conferred protection from clinical malaria, suggesting that a combination of these antigens is more effective in

vaccine development [193]. Antibodies against MSP-10, MSP-2 and MSP-7 proteins were predictive of protection from malaria in children and adults from Kenya [194]. High antibody thresholds to merozoite surface proteins are associated with protection, and development of such antibodies require longer exposure to MSPs [195, 196]. This was supported by a recent study, where anti-MSP antibodies (MSP-1-19, MSP-2, AMA-1 and EBA-175) were shown to develop in older children with greater malaria exposure [181]. These studies have further advanced the use of MSPs as vaccine candidates.

1.9.2.3 Antibody immunity to gametocytes

Gametocytes express PfEMP1, RFINS and STEVOR proteins on their surface while infecting the RBCs [106, 197, 198]. However, the role of these antigens on the gametocyte-IE-surface as targets of naturally acquired immunity has not been confirmed and remains less understood [199]. Understanding the role of these antigens as targets of antibody immunity could be useful in future vaccine studies as they could potentially help in parasite clearance and reduction of malaria transmission [170]. In a Gambian study, plasma antibodies from some children recognized antigens expressed on the surface of mature (stage V) gametocyte-IE [200]. However, it remains unclear whether these antigens were targets of IgG, as mature gametocyte-IE, normally found in peripheral blood in vivo, do not usually express antigens on the surface of the IE [199]. More recently, Ghanaian children were found to have antibodies that recognized surface antigens on mature gametocyte-IE, further supporting the role of these mature stage antigens in humoral immunity [199]. Conversely, in an older study from PNG, sera from children showed presence of antibodies against immature gametocyte stages [201], rather than mature gametocyte-IE stages.

Transmission reducing immunity (TRI) or transmission blocking immunity (TBI) is mainly observed due to exposure to antigens expressed by the gametocytes while inhabiting the human host (pre-fertilization antigens, Pfs). Pfs230 and Pfs48/45 are two of the well characterised targets of this immunity and studies highlighting their role in naturally acquired immunity have been reviewed in detail by Stone and group [202]**.** Studies by Bousema and group [203] showed that naturally acquired anti- Pfs230 and anti-Pfs48/45 antibodies develop after a malaria infection.

1.9.2.4 Antibody immunity to IE

A variety of variant surface antigens are expressed on the surface of the IE, against which naturally acquired antibodies are targeted. These antigens include RIFINs, STEVORs, SURFINs and PfEMP1s. PfEMP1 was shown to be the major target of this

antibody immunity by comparing total IgG levels against IE with PfEMP1 and IE with suppressed PfEMP1 expression, in malaria exposed individuals from Kenya [190].

1.9.2.4.1 Antibody immunity to VSAs

Early studies on antibody acquisition were targeted against the diverse variant antigens expressed on the IE surface and mostly used agglutination of IE by these antibodies as a measure of immunity [115]. They suggested that antibody responses to these surface antigens were variant-specific and were acquired only after exposure to the antigens. These findings were further corroborated by a study showing that acquisition of anti-VSA antibodies is correlated with age and exposure [204]. More recently, development of high-throughput assays to measure antibodies from plasma samples against VSAs made measurement of anti-VSA antibodies in large populations feasible, increasing the statistical power in these clinical studies [205].

In a study with adults from India, antibodies from convalescent sera against VSAs, especially PfEMP1, were shown to be both variant-specific and cross-reactive [206]. This could be attributed to similarities in the polymorphic epitopes between the different PfEMP1 variants as shown in some placental malaria studies [207, 208]. Antibody immunity to VSAs has been associated with protection from malaria in various studies, such as from Gambia [209], Gabon [210], Ghana and Sudan [211, 212]. Studies exploring the geographical differences in VSAs demonstrated that plasma antibodies from individuals from one region could recognize the antigens from a different region [78, 213]. Recently, in a study from Mali, the antibody repertoire against VSAs, including RIFINs, STEVORs and PfEMP1 was found to be lacking in children with severe malaria, suggesting an increased vulnerability to severe malaria in them [214]. This study further supported the role of antibodies against VSAs in protection from severe malaria.

Studies on antibodies against the other VSA families such as STEVORs and RIFINs are limited, however, their role in immunity against malaria cannot be ignored. Although PfEMP1 was shown to be the major VSA associated with eliciting antibody respones [190], a proportion of the antibodies were targetted against other VSAs, including RIFINs and STEVORs. In a recent study from Cameroon, children with severe and uncomplicated malaria showed presence of antibodies against the three major VSAs associated with rosetting of *P. falciparum*: PfEMP1 (NTS-DBL1α), RIFIN-A and SURFIN4.2 [101]. In a study from Gabon, the adults had high levles of antibodies against recombinant RIFIN proteins, while children showed lower levels of these antibodies [94]. The same group further showed that anti-RIFIN antibodies were long-lived, were

associated with protection from severe malaria and were associated with rapid clearance of parasites in children from these areas [95].

Studies on atibody immunity against STEVORs are very limited, with one of the studies showing high levels of anti-STEVOR antibodies only in adults, while in children no associations between these antibodies and protection from malaria were observed. These results suggest that antibodies against STEVORs could be markers of malaria exposure [215].

VSAs also include antigens expressed by gametocytes on the surface of erythrocytes, however, few studies have addressed the association of immunity to these antigens. These studies have been briefly reviewed in ‘antibody immuntiy to gametocytes’ section of this thesis.

1.9.2.4.2 Antibody immunity to PfEMP1 and severe malaria

A single PfEMP1 molecule or genotype can evade host antibodies by switching between *var* genes, that is, clonal antigenic variation [103]. Severe malaria was shown to be associated with IE capable of binding multiple receptors possibly through PfEMP1 [165]. The same study further hypothesised that simultaneous binding to multiple receptors could result in severe manifestations of the disease. Based on a serological study, parasites causing severe malaria expressed VSAs on the IE that were antigenically (and geographically) conserved and capable of being recognised by antibodies irrespective of the region the parasites originated from [213]. DC8 (transcripts of which were upregulated in severe malaria) domains were recognised by antibodies from young children from Tanzania [120] and the parasites expressing these PfEMP1 subsets (DC8 and DC13, associated with severe malaria) were shown to bind to endothelial receptors [163, 216]. Analysis from an early study demonstrated that in cases of non-cerebral, severe malaria (which requires hospitalization), immunity develops as early as the first and second infections [217].

However, the antibody repertoire against various PfEMP1 types increases with repeated exposure to malaria infections [190, 218]. It has also been suggested that a group of relatively conserved PfEMP1 variants (based on *var* gene groupings) may be associated with high pathogenicity and low host immunity [154] as IE from young children and those having severe malaria tend to be better recognized by antibodies from semi-immune children as compared to IE from older children or those with non-severe malaria [219]. As studied in children from malaria-endemic areas, antibodies to PfEMP1 domains are

acquired in an ordered fashion, with antibodies to group A PfEMP1 being acquired before those for group B and C PfEMP1 [220].

Various studies in the African and South-East Asian regions have tried to decipher the associations between antibody acquisition and malaria severity and/or the *var* genes associated with severe and uncomplicated malaria. Some of these studies have been described in this section.

In PNG, antibodies developed against PfEMP1 were shown to be acquired by children and adults, were associated with protection from symptomatic malaria and contributed to anti-malarial immunity at a young age [181]. In another study with PNG children, clinical immunity to *P. falciparum* which helped in the control of the parasite density, developed in children aged 5-9 years of age [221]. In case of Papuan adults, those with uncomplicated malaria had antibodies which specifically recognised group C PfEMP1, as well as a broad range of group A and B PfEMP1 types, suggesting that these antibodies are linked with protection from severe malaria [161].

In an early antibody study from Ghana, while levels of IgG against a recombinant PfEMP1 peptide (*var1* gene [104]) were higher in children without malaria, the antibodies did not confer protection from malaria (after correcting for age as a confounder) [211]. Although the same group later found this peptide to be inaccessible to antibodies [211], earlier antibody studies against the same peptide in Sudanese children revealed higher antibody levels in children with asymptomatic malaria compared to symptomatic malaria [222]. In later studies from Africa, antibody immunity generated against PfEMP1 was significantly linked to protection from malaria, while no such associations were observed for other IE surface antigens [190]. In Tanzanian donors, high levels of antibodies were acquired against a recombinant PfEMP1 domain belonging to group A *var* type [223].

Taken together, these studies highlight that antibodies do recognise different PfEMP1 types (either group A or B/A or others) and that these antibodies may confer protection from malaria.

1.9.2.4.2.1 Antibody immunity to DBL domains

Due to the semi-conserved structure of DBLα domains of PfEMP1, these domains have been well studied as immune targets [170]. Early studies on DBLα domains showed that semi-immune adults from Gabon had higher levels of antibodies as compared to non-immune children [224]. The expression levels of cys2 *var* genes (relative to other *var* genes) which encode DBLα domains having two cysteine residues were associated

independently with low anti-PfEMP1 antibodies, young host age and disease severity. This in turn implies that while high virulence is associated with a *var* gene repertoire least recognized by antibody immunity, the PfEMP1 molecules are also most susceptible to development of such immunity [154]. In PNG, while antibodies against recombinant DBLα domains were lower in uninfected children compared to those infected with malaria, the antibody levels were similar in adults with and without infection. The antibody levels were also shown to increase with age [225]. Recently, in young PNG children, using prospective analysis, presence of antibodies (during convalescence) against a particular DBLβ3 domain (found in conjunction with a CIDRα1 domain in the same PfEMP1) was found to reduce the risk of high-density and low-density clinical malaria [134].

1.9.2.4.2.2 Antibody immunity to rosetting phenotype

Rosetting, mediated by either PfEMP1, RIFIN or SURFIN has been implicated in protecting the IE from the host immune responses. Due to the interplay between the three VSAs, acquisition or role of antibody immunity against PfEMP1-mediated rosetting of IE in severe malaria is less understood [75]. A study by Carlson and group [226] was one of the first studies to determine the association between rosetting and cerebral malaria in Gambian children. They showed that while anti-rosetting antibodies were lacking in children with cerebral malaria, they were present in children with uncomplicated malaria, suggesting a protective role for anti-rosetting antibodies. In Kenyan children with severe malaria, no association was observed between antibodies to the IE surface and rosetting phenotype [150]. Broadly cross-reacting antibodies were associated with heterologous parasite lines as well as clinical parasite isolates from Africa having IgM-positive rosetting domains DBLα1.5 and 1.8 [127]. Antibodies against NTS-DBL1α_1 domain associated with IgM-negative rosetting, were cross-reactive against the recombinant form of the protein, but were strain-specific when the domain was expressed on the surface of an IE in the native form [227]. A study from Tanzania showed DBL2γ, also associated with rosetting [144], to be one of the first DBL domains against which anti-DBL antibodies were targeted [220]. One of the reasons for such conflicting results with PfEMP1-mediated rosetting could be that these PfEMP1s are more associated with the adherence of the parasites to host receptors, while RIFINs and STEVORs mediate binding to other uninfected RBCs [75]. Thus, antibodies directed against all three VSAs along with host factors, especially the ABO blood group, could have an impact on the disease outcome [101].

1.9.2.4.2.3 Antibody immunity to CIDRα1 domains

Studies from Africa showed that sera from children with uncomplicated malaria had higher recognition of DC8 and DC13 containing PfEMP1 as compared to PfEMP1s

without these DCs [163, 216]. In a recent controlled human malaria infection study with Kenyan adults, volunteers with low levels of naturally acquired, pre-existing antibodies detected before malaria challenge were infected with parasites with higher expression of group A and DC8-like PfEMP1 as compared to volunteers with high levels of pre-existing antibody levels [228]. In areas of high malaria transmission in Tanzania, antibodies against the EPCR-binding CIDRα1 domains of PfEMP1 were acquired by malaria exposed individuals early in life. Antibodies against these CIDRα1 domains were acquired more quickly as compared to other CIDR domains [229]. However, clear associations between severe malaria and antibody immunity to DC8 and 13 containing PfEMP1 are limited [163, 216]. In a study looking at a single severe malaria episode in children from Benin, antibodies against the EPCR binding IE strain IT4-VAR19 (containing DC8) did not show boosting from hospital presentation to follow-up period [230]. On the other hand, in Malian children with cerebral malaria or severe anaemia, while antibody repertoire against non-CD36 binding PfEMP1 was limited at hospital presentation, the antibody responses were more intense during convalescence against the non-CD36 binding PfEMP1 and PfEMP1s containing CIDRα1 domains [214].

Thus, there is evidence for the acquisition of antibody responses against the CIDRα1 domains and their role in protection from malaria in some studies. These responses were more commonly observed during the follow-up period rather than at enrolment.

1.9.2.4.2.4 Antibody immunity to other PfEMP1 domains and domain cassettes

In malaria infected children from Tanzania, levels of anti-DC4 (binding ICAM-1) antibodies increased with age [133]. In Tanzania, immunity to DC5-containing PfEMP1 were shown to be acquired early in life and provide protection against febrile malaria [137]. Similar results of early acquisition of antibodies and protection from malaria were reported for antibodies against a PfEMP1 type (PF11_0008) comprising DC5 domains in other African studies [220, 223].

These studies have increased our understanding of immunity to different PfEMP1 domains (Table 1.2). However, due to the dynamic nature of PfEMP1, identifying associations between specific PfEMP1 or PfEMP1 domains in severe malaria and antibody immunity remains challenging, especially in young children, who are most susceptible to severe malaria and mortality. Further, most of these studies are based in African regions, with antibody immunity studies in non-African regions being limited. Also, there are lower number of studies looking at associations between antibody immunity and non DC8/DC13/EPCR-binding PfEMP1.

1.9.2.4.3 IgG subclasses in immunity against malaria

There are four subclasses of IgG: IgG1, IgG2, IgG3 and IgG4. Total IgG responses against malaria have been further dissected by trying to identify which subclasses of IgG are associated with anti-malarial immunity and protection. These studies are important as IgG subclasses may be associated with certain specific antibody functions. An early review by Garraud and group [231] has highlighted studies involving the IgG subclasses. IgG1 and IgG3 seem to be the important IgG subclasses associated with different malarial antigens expressed by the merozoite, sporozoites and IE (particularly PfEMP1). A few studies describing the associations of these subclasses with immunity to malaria are highlighted below.

IgG1 and IgG3 antibody subclasses have been associated with immunity to various merozoite antigens, with IgG3 being most strongly associated with protection [232-234]. IgG2 and IgG4 subclasses targeted against a RIFIN protein RIF-29, were shown to be associated with cerebral malaria in children from Ghana [235]. IgG1, IgG3 (and IgM) were the major antibody responses mounted against CSP protein in sporozoites [183]. In vitro phagocytosis of malarial gametes mediated by opsonizing antibodies against gamete surface antigens Pfs230 and Pfs48/50, was positively correlated with IgG1 subclass [236]. In pregnant women from Malawi, high levels of IgG1 and IgG3 were observed against the CSA-binding IE and functioned by inhibiting the VAR2CSA-CSA binding [237]. Anti-VSA antibodies predominantly consisted of IgG3 subclass in children with uncomplicated malaria from Kenya [238] and children with acute malaria from Gabon [239], while during convalescence, IgG1 was shown to be the dominant IgG response. High levels of IgG1 and IgG4 against VSAs were observed in Gabonese children with uncomplicated malaria (during convalescence) and uninfected, healthy children respectively. IgG1 and IgG4 levels were associated with delays in the onset of next infection and thus clinical protection from malaria [239].

Table 1.2 Summary of *var* transcription and antibody immunity studies including binding phenotypes of different domains and domain cassettes of PfEMP1.

Domain	DC[a]	*Var* group	Binding phenotype[b]	*Var* transcription[b]	Antibody immunity against PfEMP1 domains or DC[b]
CIDRα1.1	DC8	B/A	EPCR [63]	Upregulated in SM [120, 138, 159]	Anti-DC8 Ab higher in UM [163, 216] Ab acquired in malaria exposed individuals [229] Ab levels more intense in SM during convalescence [214]
DBLβ12	DC8	B/A	gC1qR [123]	Upregulated in SM [123]	
CIDRα1.4-α1.7	DC13	A	EPCR [63]	Upregulated in SM [120, 147, 159-161]	Anti-DC8 Ab higher in UM [163, 216] Ab acquired in malaria exposed individuals [229] Ab levels more intense in SM during convalescence [214]
DBLβ1/β3	DC13	A	ICAM-1 [125]	Upregulated in SM [135]	Anti-DBLβ3 Ab protects from clinical malaria [134]

DBLβ3	DC4	A, B/A	ICAM-1 [133]	Upregulated in SM [135]	Anti-DC4 Ab levels increased with age [133]
DBLβ-C2/DBLβ5		B, C	ICAM-1 [131, 136]		
CIDRα2-6		C	CD36 [129, 130]		
	DC5		PECAM1 [137]	Upregulated in SM and cerebral malaria [120, 141, 161]	Ab protect from febrile malaria [137, 220, 223, 240]
DBLζ5/ DBLε4	DC6		IgM, α2-macroglobulin, IgM+ rosetting [127, 139, 140] for DBLζ/ε domains.	Upregulated in SM [138]	
DBLζ2/ DBLε3/ DBLε11	DC11		IgM, α2-macroglobulin, IgM+ rosetting [127, 139, 140] for DBLζ/ε domains.	Upregulated in SM [123, 141]	
NTS-DBL1α		A	Rosetting: CR1, heparan sulfate, blood group A antigen [142, 143]	Upregulated in SM [149]	Cross-reacting Ab against recombinant domain, strain specific Ab against native domain expressed on IE [227]
DBL2γ		A	Rosetting [144]		Ab protect from malaria fevers [223] >60% children had Ab, one

				of the first DBL domains to induce Ab responses [220]
DBLα1.5 /1.8	A	IgM+ rosetting [127]		Cross-reacting Ab against clinical rosetting parasites [127]
CIDRβ/γ/δ	A	Rosetting [61, 127]		
DBLα	A		Upregulated in SM [120, 153, 154]	Ab in malaria > Ab in uninfected [225]

[a] The domains assigned to the DC can also be found outside the defined DC.

[b] The binding phenotype, *var* transcription and antibody immunity studies are described for the particular domain or domain type or similar domains and have not necessarily been linked to the accompanying specific DC or *var* group. Studies from different geographical locations are included, not all findings listed.

PfEMP1, *Plasmodium falciparum* erythrocyte membrane protein 1; DC, domain cassette; CIDR, cysteine-rich inter-domain region; EPCR, endothelial protein C receptor; SM, severe malaria; Ab, antibody; UM, uncomplicated malaria; DBL, duffy binding-like; ICAM-1, intercellular adhesion molecule 1; CD36, cluster of differentiation 36; PECAM1, platelet/endothelial cell adhesion molecule.

1.10 Role of malarial antibodies

Naturally acquired, anti-malarial antibody immunity has varied functions and is targeted against different stages of the parasite life- cycle. These antibodies are directed against the proteins expressed during the blood stages, such as the merozoites, gametocytes or the IE and the pre-erythrocytic sporozoites (reviewed in [241]).

1.10.1 Role of antibodies against sporozoites

CSP protein is the major target of anti-sporozoite immunity and the major vaccine candidate for malaria infection. However, functional mechanisms underlying vaccine-induced immunity or antibody immunity to CSP in general remain less studied [183]. In an in vitro study, antibodies against CSP were shown to neutralize the motility of sporozoites (from host dermis to blood vessel) [242]. Functional antibodies against sporozoites were also shown to inhibit the traversal of sporozoites into the liver [243]. Recently, antibodies targeted against the central repeat region of CSP were shown to activate the complement on the sporozoites in vitro. This led to inhibition of the passage of sporozoites through the liver, thereby limiting the liver-stage infection and causing death of the sporozoites [183]. The same study further demonstrated acquisition of these complement-fixing antibodies to be age-dependent and associated with protection from malaria.

1.10.2 Role of antibodies against merozoites

Various functional roles have been attributed to antibodies against merozoites. Antibodies against merozoite surface antigens such as MSP-1 and MSP-2 help in complement fixation which in turn leads to inhibition of merozoite invasion of RBCs and its replication therewith. This leads to protection from clinical malaria and high density parasitaemia in children [244]. EBAs and PfRH are two other groups of invasion ligands necessary for merozoite invasion of RBCs and targets of invasion inhibitory antibodies [245]. In children with symptomatic malaria, levels of invasion blocking antibodies -which function by blocking EBA-175 binding to its receptor glycophorin A- were associated with protection [246].

Another function associated with anti-merozoite antibodies is antibody-dependent cellular inhibition (ADCI), where merozoites that have been opsonized by antibodies, interact with different monocytes and macrophages to release soluble factors which in turn lead to inhibition of parasite growth [247]. MSP-1 [248], MSP-2 [249], MSP-3 [250] and GLURP [251] have been shown to be the major targets of these antibodies in various studies.

Antibody-dependent respiratory burst (ADRB), which occurs due to production of reactive oxygen species (ROS) by neutrophils interacting with opsonized merozoites has been linked to protection from clinical malaria [252]. MSP-5 was shown to be the target for these antibodies in one study [253] and ADRB was shown to facilitate parasite clearance in another study [254].

Merozoites opsonized by acquired antibodies can be directly phagocytosed by monocytes [255]. Such opsonizing antibodies reduce the risk of clinical malaria, as shown in studies from Kenya [193] and PNG [255]. MSP-2, MSP-3 [193], MSPDBL1 [256] and MSPDBL2 [257] have been shown to be the targets of these opsonizing antibodies.

1.10.3 Role of antibodies against gametocytes

Transmission reducing immunity (TRI) is one of the important functions attributed to antibodies targeting various gametocyte stages during their formation, maturation and transmission [202]. This antibody immunity can function even within the mosquito midgut by inhibiting fertilization of the gametes, reducing cell-cell contact between the micro and macro gametes, opsonization followed by lysis or lysis mediated by activation of the complement system (reviewed by [202])**.** In young children from Ghana enrolled in a prospective study, antibodies against the surface antigens expressed by mature gametocyte-IE detected at enrolment were shown to decrease the levels of smear-detectable (patent) gametocytes in subsequent visits [199]. Based on anti-gametocyte-IE immunity studies in PNG, it was proposed that immunity to the gametocyte surface antigens during the immature stages may affect the gametocytes' further maturation, thereby influencing malaria transmission [201]. While in vitro phagocytosis of malarial gametes was shown to occur in the presence of anti-Pfs230 and anti-Pfs48/45 antibodies, in vivo phagocytosis of intraerythrocytic gametocytes, mediated by naturally acquired, transmission-blocking antibodies did not occur [236].

1.10.4 Role of antibodies against asexual IE

Antibodies acquired against the various antigens expressed on the surface of the IE, specially the PfEMP1 protein have been shown to function by inhibiting the adhesion of the parasite to various host receptors, inhibition of rosetting and opsonic phagocytosis of the IE by monocytes (reviewed in [170, 241]). In one study, anti- PfSEA-1 (*Plasmodium falciparum* schizont egress antigen-1) antibodies were shown to function by arresting the schizont rupture, thus decreasing the parasite replication and blocking its egress from the RBCs. This in turn was thought to confer protection from severe malaria in Tanzanian children [258].

1.10.4.1 Inhibiting cytoadhesion

Cytoadhesion and sequestration of the IE to host endothelial receptors is one of the major pathophysiological causes of severe malaria. One of the functions of naturally acquired immunity is to disrupt this adhesion, which has been demonstrated in numerous studies.

Antibodies against various domains of VAR2CSA -the PfEMP1 associated with placental malaria- have been shown to inhibit the VAR2CSA-CSA adhesion in various studies [237, 259] and associated with improved birth outcomes [260].

Studies on role of antibodies in inhibiting parasite-receptor adhesion in non-pregnant individuals are few and limited. Antibodies purified from individuals from malaria endemic areas against recombinant EPCR-binding CIDRα1 domains can inhibit the binding of the domains to EPCR [128]. Anti-DC4 antibodies were capable of inhibiting adhesion of DC4-containing PfEMP1 to ICAM-1 receptor [133]. A recent study from Lennartz and group [135] showed that IgG from Liberian adults' plasma with malaria have the capacity of inhibiting the binding of ICAM-1 and PfEMP1 having specific DBLβ motifs. The importance of anti-DBLβ antibodies was supported in a study using sera from Ghanaian children with cerebral malaria, where the antibodies could inhibit the binding of DBLβ domains to ICAM-1. Similar inhibition was observed for antibodies from rats immunized with DBLβ domains, further suggesting the cross-reactive nature of these antibodies [261]. Monoclonal antibodies could partially inhibit and reverse binding of laboratory derived parasite lines to ICAM-1 and CD36 [262].

Thus, so far, studies have shown anti-PfEMP1 antibodies to inhibit binding of PfEMP1 or PfEMP1 domains to CSA, EPCR, ICAM-1 and CD36.

1.10.4.2 Opsonic phagocytosis

Opsonic phagocytosis, that is, uptake of IE by monocytes and macrophages opsonized by antibodies is an important function attributed to anti-malarial antibodies. One of the earliest studies demonstrating the opsonic activity of antibodies in monocyte mediated phagocytosis of *P. falciparum* was by Bouharoun and group [263]. Opsonic antibodies were shown to mainly target PfEMP1 on the surface of the IE, thus facilitating parasite clearance [190]. This antibody function has been widely studied in pregnancy associated malaria. High levels of opsonizing antibodies were associated with parasite clearance and lower anaemia in Malawian pregnant women [264]. Opsonic phagocytosis was shown to be hampered by HIV infection in pregnant women from Malawi [265] and Kenya [266]. Recently, opsonic antibodies against specific VAR2CSA domains were shown to be IE

strain-specific [267]. Fewer studies have investigated the role of opsonizing antibodies in non-pregnant individuals or children with severe malaria. One study demonstrated the association of high opsonic antibody levels (against IE with different binding phenotypes) with reduced parasitaemia during follow-up in adults with malaria [268]. Opsonic phagocytosis was shown to improve in the presence of complement fixation [269] and was associated with the release of cytokines [270].

1.10.4.3 Inhibiting rosetting of IE

Antibodies to recombinant, extra-cellular, rosetting strain-specific domains, particularly the NTS-DBL1α domain could inhibit rosetting, impact surface reactivity and opsonise the IE for phagocytosis [127, 271, 272]. However, such functional responses against rosetting IE were found to be strain-specific [271]. Sub-domain 3 (SD3) loop of NTS-DBL1α domain of PfEMP1 was shown in one study to be the target of rosette-inhibiting antibodies [273]. As demonstrated in a recent study, these antibodies could also be targeting the DBL2γ domain of PfEMP1 [101].

Studies on acquisition of antibodies and functional antibodies in cases of severe malaria, particularly in children are limited. A more detailed understanding of such immunity is required, as acquired antibody immunity may not necessarily correlate with protection from malaria [241]. Recently, a prospective study involving children from Kenya suggested that a combination of different antibody-dependent functions against different parasite targets, was necessary to provide protection from severe malaria [274].

1.11 Malaria in pregnancy

Pregnancy associated malaria (PAM) caused by *P. falciparum*, affects both mother and child. It is most prevalent in women in their first and second trimester and mainly observed in sub-Saharan Africa [275]. Although pregnancy associated malaria is mostly asymptomatic [276], it can lead to maternal anaemia, still birth and low birth weight [275].

1.11.1 VAR2CSA protein

Placental malaria caused by *P. falciparum* is mediated through the parasites' VAR2CSA protein, a serologically distinct PfEMP1 expressed by the *var2csa* gene [113]. This protein sequesters the IE to CSA [65, 66] on syndecan-1, a chondroitin sulfate proteoglycan present in the placental syncytium [277]. As reviewed in [278], VAR2CSA protein consists of multiple DBL domains and interdomain regions with DBL 2, 3, 5 and 6 domains associated with CSA binding. However, several studies have tried to narrow down the exact domains associated with CSA binding and as targets of antibodies and

identified the N-terminal region of VAR2CSA, in particular, the interdomain 1 and DBL2X region to be most important (reviewed in [278]). Unlike other PfEMP1 molecules, *var2csa* was shown to be lacking the CIDR and DBLγ domains [113]. *Var* genes other than *var2csa* and belonging to groups A, B and C *var* types were also shown to be transcribed in pregnant women, suggesting simultaneous binding of the parasites in the placenta and other endothelial linings [279].

1.11.2 Antibody immunity to placental malaria

VAR2CSA is the major target of antibody immunity in PAM [280]. Antibodies to VAR2CSA are acquired in a parity dependent manner [281] by pregnant women living in malaria endemic areas with stable transmission and are capable of blocking the IE-CSA binding [282]. Various studies have demonstrated the role of these antibodies in protection from placental malaria through reduction of parasitaemia [283] and inhibition of binding of parasite IE to CSA [284]. Such inhibition antibodies were also associated with increased birthweight of newborns as well as their gestational age [260]. Recently, the DBL2 region of VAR2CSA along with its adjacent interdomain regions and DBL4 were shown to be the targets of adhesion-inhibitory antibodies [285], while DBL1-DBL2 domains were the antibody targets associated with reduced risk of low birthweight [278]. Opsonising antibodies also play an important role in conferring protection from placental malaria and poor pregnancy outcomes and have been discussed briefly in ‘role of antibodies against IE’ section (section 1.10.4.2).

1.11.3 Preventing malaria in pregnancy

Currently, WHO recommends use of insecticide-treated nets (ITNs) in combination with intermittent preventive treatment in pregnancy (IPTp) and sulfadoxine-pyrimethamine for HIV-negative pregnant women for preventing PAM in malaria endemic and high transmission regions. For HIV-positive women, co-trimoxazole is used instead of sulfadoxine-pyrimethamine. In low transmission areas, PAM control and prevention is achieved through screening of asymptomatic women, early detection (first antenatal visit) and distribution of ITNs [286].

1.12 Anti-malarial treatment and drug resistance

Based on WHO guidelines [287], a good anti-malarial treatment should guarantee complete cure, reduce transmission, and prevent drug resistance. Currently, artemisinin-based combination therapies (ACT) are used for treatment of severe and uncomplicated malaria. The difference in the treatment of the two disease severities lies in the dosage and mode of administration. Artemisinin and its derivatives were found to be better at

treating severe malaria as compared to quinine-based drugs [288, 289]. ACTs include the following partner drugs: lumefantrine, amodiaquine, piperaquine, mefloquine, pyronaridine and sulfadoxine-pyrimethamine (SP). Primaquine is also included in the treatment regimen in low malaria transmission areas. ACT is also used for the treatment of *falciparum* malaria during pregnancy for women in their second and third trimester. For women in their first trimester, quinine with clindamycin is recommended. Treatment of *P. vivax* includes chloroquine and primaquine (after testing for glucose-6-phosphate dehydrogenase deficiency). Recently, tafenoquine [290] was approved as a drug for eradication of *P. vivax* hypnozoites.

Drug resistance in malaria treatment has always been a challenge. Drug resistance is said to develop when parasite clearance rate slows down, and abatement of fever requires a longer time [291]. Following the treatment guidelines put forth by WHO is important in preventing resistance.

1.13 Vaccines against malaria

The World Health Organization aims to develop a vaccine against malaria by 2030, which has at least 75% protective efficacy against clinical malaria [292]. Numerous challenges need to be overcome before the development of such a vaccine. Current vaccine development is impeded by limited knowledge on antigens that can be used as targets for vaccines, antigens as correlates of protection and the mechanisms through which immunity can confer protection [293].

RTS,S (RTS,S/AS01) is currently the most advanced vaccine candidate that has been developed against human malaria. The vaccine is based on the central-repeat and C-terminal regions of CSP (reviewed in [294]) which induces both antibody and CD4+ T cell responses [295]. However, during the phase III clinical trial, this vaccine was found to be only partially efficacious against clinical malaria with protection waning with time [296].

Whole sporozoites that have been attenuated, are aseptic and have been purified and cryopreserved (PfSPZ) are also being studied as vaccine candidates and are undergoing clinical trials [180]. These vaccines were shown to function through PfSPZ-specific antibody and T-cell responses. Vaccines targeting the liver stage antigens of the parasites and eliciting strong CD8+ T-cells are also potential candidates [297].

Attenuated *Plasmodium* parasites, both during the liver stage and blood stage using chemical attenuation are also being explored as potential vaccine candidates [298].

Antigens expressed by other stages of the parasites are also major candidates for a vaccine. These include antigens expressed by merozoites, gametocytes and the infected erythrocytes. I will briefly discuss some of these antigens as vaccine candidates and the challenges involved in using PfEMP1 as a vaccine candidate.

PfAMA1, is a polymorphic merozoite antigen. A cocktail comprising of three recombinant variants of this protein has been used as an immunogenic vaccine and is currently under phase I clinical trial [299]. The other merozoite antigen identified as a strong vaccine candidate is the *P. falciparum* reticulocyte-binding protein homolog 5 (PfRH5), which is required for erythrocyte invasion of the parasite. These vaccines were shown to be efficacious in *Aotus* monkeys [300] and humanized mouse models [301] and were associated with anti-PfRH5 antibody concentrations.

Transmission blocking vaccines (TBV) using antigens expressed by the gametocytes are not associated with protection from clinical symptoms, but with prevention of further malaria transmission as they target the parasite's life-cycle in the mosquito [302]. The most advanced vaccine targets for TBV are the Pfs25 (ookinete surface protein) and Pfs48/45 and Pfs230 (gametocyte antigens). Transmission blocking activity by these antigens was shown to be dependent on IgG levels [303, 304]. While Pfs25 is under phase I trial [305], Pfs48/45 is under pre-clinical trials (reviewed in [302]).

Given the polymorphic nature of *P. falciparum* antigens, a combination vaccine, comprising of vaccine candidates from different parasite stages would be beneficial. While one such synergistic vaccine involving the RTS,S/AS01 (sporozoite) and ChAd63-MVA ME-TRAP (liver-stage) was not associated with improved protection [306], a combination of pre-erythrocytic and transmission blocking vaccines showed more efficacy in reducing parasite densities as compared to individual efficacies [307].

1.13.1 PfEMP1 as vaccine candidates

PfEMP1 could be a potential vaccine candidate as it is an important immune target, especially for antibody immunity [190]. PfEMP1, along with other surface antigens are implicated in the pathogenesis of severe disease because it mediates sequestration to various host receptors and resulting organ-specific damage (reviewed in [145]).

VAR2CSA is the most well defined PfEMP1 protein and has been implicated in placental binding of the malaria parasite [113]. Unlike other PfEMP1 proteins, VAR2CSA is encoded by a relatively more conserved *var* gene [113], which could be the reason for its progression to human clinical trials as a vaccine candidate [308]. PAMVAC is one of the

two vaccines in phase I trial [309], where a 73 kDa fragment of VAR2CSA is the target of inhibitory and cross-inhibitory antibodies [310]. PRIMVALVAC is the other vaccine candidate, where a 105 kDa fragment of VAR2CSA is targeted [309].

Early studies using animal models have shown that combinations of various PfEMP1 domains, such as different CIDRα domains, resulted in the generation of broadly cross-reactive antibodies against them [311], while the NTS-DBLα domain combination resulted in acquisition of cross-reactive antibodies capable of reducing the IE-mediated sequestration [312]. The production of cross-reactive antibodies against PfEMP1 and other VSAs was further supported in a controlled human malaria infection trial with malaria naïve volunteers [313].

The biggest difficulty in using PfEMP1 proteins as vaccine candidates lies in their antigenic diversity and variability [90, 110, 114]. Thus, most of the vaccine studies with PfEMP1 have relied on using combinations of its different domains, which in turn can induce cross-reactive antibodies against different PfEMP1 variants. Conserved epitopes expressed on the PfEMP1 protein could also be used as targets of immune responses. Limited knowledge on conserved sets of PfEMP1 domains capable of eliciting protective antibody responses, the tertiary and quaternary structure of PfEMP1 [170], together with difficulty in the expression of correctly folded, soluble, non-glycosylated recombinant proteins as targets which can elicit antibody responses [314] have so far impeded the progress of this field.

PfEMP1 based vaccines are required to confer long-lasting antibody protection which can be boosted by further infections. The success of developing such a vaccine lies in understanding the acquisition of antibody responses against PfEMP1 proteins, their boosting and maintenance as well as the identification of functional mechanisms underlying anti-PfEMP1 immunity [170].

1.14 Gaps in literature

PfEMP1 is an important immune target. Various studies have tried to decipher its role in severe malaria, uncomplicated malaria and placental malaria. Studies measuring the transcript levels of different PfEMP1 types and domains are abundant. Most studies identifying PfEMP1 domains as targets of immunity have relied on laboratory-based parasite lines with few studies using clinical isolates from individuals with severe or uncomplicated malaria. Studies on PfEMP1 as a target of antibody immunity, particularly in young children with severe malaria are limited [134, 214, 226]. Many studies have improved our understanding of functional roles of these antibodies (adhesion-inhibitory,

rosette disrupting and opsonizing antibodies). However, studies linking functional antibody levels with severe malaria in children and their associations with protection are limited. Understanding immunity to PfEMP1 types in young children is important as PfEMP1 is one of the major causes of severe malaria. Also, children are most susceptible to complications arising from severe malaria leading to death. Most of the studies highlighted in the review also indicate the absence of sufficient data on antibody immunity to PfEMP1 in non-African regions such as the Asia- Pacific countries. Studying antibody immunity to surface antigens of the IE, particularly PfEMP1 protein is important as it will help us understand the pathogenesis of severe malaria in malaria endemic regions and contribute towards PfEMP1 vaccine development.

2 Chapter 2: Thesis overview

The overall hypothesis for the study is that antibody responses to different subsets of PfEMP1 types differ between individuals with severe malaria and uncomplicated malaria (disease severity) and/or between hospital presentation and convalescence (disease stage). I hypothesise that children with severe malaria tend to have low levels of antibodies to PfEMP1 at hospital presentation as compared to children with uncomplicated malaria at presentation, with the antibody levels developing during the follow-up period/convalescence. Further, I hypothesise that severe malaria in adults is associated with a lower antibody response compared to adults with uncomplicated malaria. Finally, I hypothesise that in severe malaria cases, the antibody profile in adults may differ from the antibody profile observed in children.

Based on these hypotheses, the specific aims for this thesis are as follows:

1. To investigate if recombinant CIDR domains of PfEMP1 are targets of antibody immunity in severe or uncomplicated malaria cases in children from Papua New Guinea (Chapter 4).

To investigate recombinant CIDR domains of PfEMP1 as targets of immunity in chapter 4, antibody levels against these domains were measured in plasma collected from Papua New Guinean (PNG) children with either severe or uncomplicated malaria at two time points: hospital presentation (acute) and 8 weeks later (convalescence/follow-up). Age-matched community controls were also included in the study. Antibody levels were measured against 35 recombinant CIDR domains and included domains which were known to or predicted to bind to endothelial protein C receptor (EPCR), CD36 or associated with rosetting of the infected erythrocytes (IE).

2. To investigate if functional (opsonising) antibodies against parasite IE expressing the entire native PfEMP1 (representing different PfEMP1 types) differed between children with severe or uncomplicated malaria in Papua New Guinea (Chapter 5).

In chapter 5, to determine differences in functional antibodies (by disease severity) to whole parasites expressing the native PfEMP1 molecule in the same PNG population as used in chapter 4, an opsonic phagocytosis assay was employed. Levels of opsonising antibodies were measured against parasite IE lines representative of four PfEMP1 types: an ICAM-1 binder (which also binds CD36), a CD36 binder, an EPCR binder and IE expressing PfEMP1 associated with rosetting.

3. To use sequences of PfEMP1 domains associated with severe or uncomplicated malaria and express them using wheat germ cell free protein synthesis system (WGCFS) and to evaluate these PfEMP1 domains as targets of immunity in malaria exposed individuals from Papua, Indonesia and Malawi (Chapter 6).

In chapter 6, PfEMP1 domains, whose transcripts were upregulated in severe or uncomplicated malaria cases from Papua, Indonesia, were cloned and expressed using the WGCFS. Antibody levels against 32 PfEMP1 domains thus generated were measured in two study populations. The first population from Papua, Indonesia, was the same as the one which was used to determine the differential *var* gene expression (we had more number of plasma samples for antibody measurements than were used for *var* transcription). Plasma collected at hospital presentation were used for antibody measurements. The second population included children from Malawi having either cerebral or uncomplicated malaria. Samples had been collected at two time points: hospital presentation and 4 weeks later. Age-matched community controls were also included in the study.

Results for each aim are presented in chapters 4, 5 and 6 respectively. Each chapter has a brief introduction, details of the study populations used, and the statistical analyses, followed by the results and a detailed discussion. General experimental procedures used for each aim have been described separately in the materials and methods chapter.

For chapter 7, the main findings from the study have been discussed along with the overall limitations, strengths and potential future studies that can be carried out.

3 Chapter 3: Materials and methods

3.1 Study populations

Three study populations were used to measure antibody levels against different PfEMP1 domains or PfEMP1 types. The first population was from PNG and included children with severe malaria or uncomplicated malaria at hospital presentation and in convalescence and age-matched community controls. This population was used for aim 1 and aim 2 (chapter 4 and 5 respectively). The second population included Papuan adults with severe or uncomplicated malaria at hospital presentation only. The third population was from Malawi and included children with cerebral malaria or uncomplicated malaria at hospital presentation and in convalescence along with community controls. The Papuan and Malawian study populations were used for aim 3 (chapter 6). Details for the study populations have been described in the study population section of the respective results chapters.

3.2 Parasite culture

3.2.1 Maintenance

Erythrocytes infected with *P.falciparum* were grown in a 150 X 15 mm (25 ml) or 100 X 15 mm (10 ml) petri dish (Falcon, Corning) and were provided daily with RPMI-HEPES complete medium (Medium Preparation Unit, Peter Doherty Institute for Infection and Immunity, University of Melbourne) containing 0.2% w/v sodium bicarbonate and supplemented with 0.25% Albumax II (Gibco, Life Technologies) and 5% heat inactivated (at 57^{o}C for 60 minutes) human sera (HIHS: pooled from healthy Melbourne donors having different blood types (Australian Red Cross Blood Services, West Melbourne). O positive, red blood cells (from Australian Red Cross Blood Services, West Melbourne) were added and the cultures were maintained at a parasitaemia of 2-8%. The culture plates were stored in a box gassed with a mixture of gasses (5% carbon dioxide, 1% oxygen and 94% nitrogen) and incubated at 37^{o}C.

Percent parasitaemia was calculated by counting the ring or trophozoite stage parasites using thin smears on glass microscope slides stained with Giemsa (Merck) (10% v/v made in tap water). Slides were read under a light microscope using the oil immersion lens. Total number of RBCs were counted in one field and IE were counted in 10 fields, which was then averaged to determine the % parasitaemia.

3.2.2 Freezing infected erythrocytes (IE)

Erythrocytes infected with ring stage parasites were resuspended and spun at 490 x g for 5 minutes at room temperature (RT). Two times the pellet volume of sterile, RT glycerolyte (Fenwal, 57% glycerin, 16 grams/litre sodium lactate, 300 milligrams/litre potassium chloride buffered with monobasic and dibasic sodium phosphate, pH6.8) was added dropwise, in two steps: first added one third of the total volume and then added the remaining volume. The pellet was resuspended gently and 400 µl were transferred into sterile, 1ml cryotubes (Thermo Scientific). The tubes were initially stored in an isopropanol freezing container at -80^{o}C (24-48 hours) and later transferred to -80^{o}C freezer or liquid nitrogen.

3.2.3 Thawing infected erythrocytes (for ring stage)

A vial of frozen IE was thawed in the 37^{o}C water bath for 1-2 minutes. Next, 0.1 times the pellet volume of sterile, 12% sodium chloride (NaCl) solution was added drop wise, followed by 10 times the pellet volume of 1.6% NaCl solution. Pellet was collected after centrifuging at 490 x g for 5 minutes at RT and resuspended using 10 times the pellet volume of 0.9% NaCl-0.2% glucose solution. The solution was centrifuged, and pellet was resuspended using RPMI-HEPES complete medium, RBCs as required, and maintained as described above.

3.2.4 Thawing infected erythrocytes (for trophozoite stage)

Vials with frozen trophozoites were thawed in the 37^{o}C water bath for 1-2 minutes. Equal volume of sterile malaria thawing solution (MTS: 3.5% NaCl made in phosphate buffer saline (PBS)) was added dropwise followed by another 2 ml of MTS. Pellet was collected after spinning at 313 x g for 4 minutes at RT. The MTS wash was repeated and further washed, first with 2 ml of 50:50, MTS: PBS solution followed by PBS only. Pellet collected after the washes was resuspended with RPMI-HEPES complete medium and the required amount of RBCs and maintained as described previously.

3.2.5 Synchronisation of *P. falciparum* (for ring stage)

IE with at least 5% rings were resuspended and spun at 490 x g for 5 minutes at RT. The pellet was resuspended with 10 times the pellet volume of sterile, pre-warmed 5% w/v sorbitol (made using MilliQ water) (Ajax Finechem, Thermo fisher Scientific) and incubated for 5 minutes in a 37^{o}C water bath. The pellet then collected was resuspended in RPMI-HEPES complete medium and required amount of RBCs and maintained at required conditions.

3.2.6 Enrichment of *P. falciparum* trophozoites by gelatin floatation

Cultures of IE, at least 5% mature trophozoites, in RPMI-HEPES complete medium were resuspended, spun down as previously described and the pellet was resuspended with pre-warmed 0.75% w/v gelatin (Sigma) made in RPMI-HEPES wash buffer (without Albumax and heat inactivated human serum) to get a cell suspension of 10%. The suspension was incubated in a tube in a 37^{o}C incubator for 45 minutes. The supernatant containing the knobby parasites was collected using a transfer pipette and washed once with 10-20 ml RPMI-HEPES wash buffer at 490 x g, RT for 5 minutes. The pellet obtained was then resuspended in RPMI-HEPES complete medium and RBCs and maintained as described previously.

3.2.7 Enrichment of *P. falciparum* rosette formation by gelatin-heparin floatation

The initial steps in this process are similar to enrichment of trophozoites as described above, where, using a vacuum-aspirator, the medium was removed from the parasite culture plate while keeping the parasites untouched. Next, 0.75% gelatin was added to the culture to get a 10% cell suspension, and this was incubated in a 37^{o}C incubator for 45 minutes. Non-rosetting trophozoites float in the supernatant while the rosetting ones settle in the pellet. The supernatant was discarded, and the pellet was then resuspended with 0.75% gelatin containing 100 units/ml of heparin lithium salt (from porcine intestinal mucosa, Sigma Aldrich, product code H0878-100KU), such that the final cell suspension was 10%. This was incubated once again at 37^{o}C for 45 minutes. The heparin disrupts the rosettes, and the rosetting trophozoites now float in the supernatant, which was collected and washed with RPMI-HEPES wash buffer at 490 x g, for 5 minutes at RT (this wash step removes the heparin from the solution to facilitate rosetting of the IE once in culture). The pellet thus obtained was resuspended in RPMI-HEPES complete medium and RBCs and maintained at appropriate conditions.

3.2.8 Purification of *P. falciparum* trophozoites using a Percoll gradient

IE with 5-8% trophozoites were resuspended and spun at 490 x g for 5 minutes at RT. The pellet was resuspended with an equal volume (as the pellet) of RPMI-HEPES wash buffer. In a 15 ml tube, sterile pre-warmed Percoll solutions (GE Healthcare) (made in RPMI-HEPES without sodium bicarbonate) were layered carefully, starting with 3 ml of 80%, followed by 2ml each of 60% and then 40%. The gradient was overlayed with the resuspended parasites solution while taking care not to disrupt the Percoll layers. The tubes were spun at 1620 x g for 15 minutes at RT. The rings and uninfected RBCs pellet in the 80% layer, while the trophozoites settle in the 60% layer. The trophozoites were

collected in a separate tube by careful aspiration using a transfer pipette and washed twice with RPMI-HEPES wash buffer at 490 x g for 5 minutes at RT. Purity was checked by making thin smears of the trophozoites and Giemsa staining. This yields >70% purity of trophozoites, which are then usually resuspended in wash buffer, ready to be used.

3.2.9 Receptor binding assay

Binding phenotypes of the different parasite lines/strains commonly used in the lab were routinely verified using the receptor binding/adhesion assay. Receptors were tested in triplicates and included: CSA (from bovine trachea, Sigma Aldrich), CD36 (R&D Systems) and ICAM-1 (from transfected chinese hamster ovary, Bender MedSystems). No-receptor control was used as negative control. For each receptor, the inside of a 10 ml plastic petri dish was marked with three circles using a Dako immunohistochemistry wax pen. ~7-10 µl of CSA (100 µg/ml), CD36 (20 µg/ml) and ICAM-1 (8 µg/ml), diluted in PBS was added to the centre of each receptor circle. This plate was incubated at 4^{o}C, overnight (O/N) in a humid box or in a 37^{o}C incubator for 2 hours. Non-specific binding was blocked with 50 µl of sterile 1% casein (in PBS, Thermo Scientific) added over each receptor spot. Incubation was for 30-60 minutes in a 37^{o}C incubator. IE with at least 5% trophozoites were enriched using the gelatin floatation method as previously described and the pellet was resuspended in cytoadherence medium (CAM: RPMI-HEPES, pH 6.8, without sodium bicarbonate with 10% HIHS) to get a 5% haematocrit. The receptor plate was then washed thrice: by adding sterile PBS to the plate, gently swirling the contents and discarding the solution using a vacuum-aspirator. 45 µl of the parasite cell suspension were added to each receptor spot and incubated in a gassed box at 37^{o}C for 1 hour. Unbound cells were washed 3-4 times with sterile PBS. The bound cells were fixed with 2% glutaraldehyde (made in PBS) O/N, stained with 10% Giemsa stain for 10 minutes and counted using a light microscope under 40X lens (400 times magnification). Three fields per receptor spot were counted, averaged and cells per mm^2 calculated.

3.2.10 Selection of IE for receptor binding

The E8B parasite line was selected for ICAM-1 binding using this assay as described previously [315]. A 10 ml petri dish was coated with 1 µl spots of ICAM-1 (8 µg/ml) and incubated O/N at 4^{o}C under humid conditions. Plate was blocked with 1% sterile Casein for 30 minutes at 37^{o}C and washed thrice with sterile RPMI-HEPES wash buffer using the vacuum suction. IE with >6% trophozoites were enriched using the gelatin floatation method, resuspended in 10 ml CAM and added to the receptor coated plate. These were incubated at 37^{o}C in a gassed box for 1 hour. The unbound cells were washed 4-6 times with sterile RPMI-HEPES wash buffer. RPMI-HEPES complete medium was then added

along with required amount of RBCs. The cultures were then maintained as previously described.

3.2.11 Testing and treating mycoplasma contamination

The parasite cultures were routinely tested for mycoplasma contamination using the manufacturer's instructions from the Lonza MycoAlert™ kit. Briefly, 1 ml of resuspended parasite culture was spun at 200 g for 5 minutes at RT. 100 µl of the supernatant along with 100 µl each of positive (from pooled mycoplasma positive parasite strains) and negative controls (Lonza assay buffer) were transferred to Nunc 96-well black plates. 100 µl of the MycoAlert™ Reagent was added. After 5 minutes incubation at RT, luminescence was measured using the FLUOstar Omega luminometer (BMG Labtech). This was assigned as 'Read A'. 100 µl MycoAlert™ Substrate was then added to each sample and incubated at RT for 10 minutes. Luminescence was measured once more and was now called 'Read B'. Ratio of Read B: Read A was calculated. A ratio of < 0.9 indicates no mycoplasma contamination, 0-9-1.2 suggests that there may be contamination and the culture needs to be re-tested, while a ratio > 1.2 means cultures are positive for mycoplasma contamination. Cultures positive for mycoplasma were treated with 5 µg (per 10 ml culture) of Mycoplasma Removal Agent (MRA: MP Biomedicals, 50 µg/ml) for one week and were retested before using for further experiments.

3.2.12 Cloning of *P. falciparum*

Highly synchronous, 2-5% trophozoite stage IE were used. Cells /ml was calculated using the haemocytometer and number of IE was calculated by multiplying the % parasitaemia to the cell count. The parasites were then diluted using RPMI-HEPES with 10% HIHS (cloning medium) to get three parasite dilutions: 100 IE/ml, 10 IE/ml and 3 IE/ml. Fresh RBCs (at least 3 weeks before expiration) were added to each dilution (1 drop/ml). 100 µl of one dilution/ well were added to multiple wells of a 96-well plate. Similarly, 100 µl of the other dilutions were added, to get 10 parasites/ well, 1parasite/well and 0.3 parasite/well respectively. The plates were incubated in a gassed box at 37^{o}C. Medium was changed every two days and after a week, parasitaemia was checked for each dilution using thick smears. Parasites first appear/come up in the 10 parasites/well dilution after ~ 1 week of culturing followed by the 1 parasite/ well a couple of days later. Once the parasitaemia started increasing, parasites from the 1 parasite/well dilution were transferred, first to a 6 well plate (4 ml culture) and then to a 10 ml culture plate. The cloning medium was used for a few more replication cycles before switching back to RPMI-HEPES complete

medium. The binding phenotype of the parasite clone was checked using the receptor binding assay as described previously.

3.3 THP-1 culture

3.3.1 Maintenance

Undifferentiated THP-1 cells are non-adherent, pro-monocytic cells (obtained from ATCC Manassas, VA, USA), which were maintained in RPMI 1640 medium (Gibco, Life Technologies) containing 10%, heat inactivated Foetal Bovine Serum (FBS: Gibco, Life Technologies), 1% Penicillin-Streptomycin-Glutamine (PSG: Gibco, Life Technologies, 100X) and 25 mM HEPES (Gibco, Life Technologies). Every two days the culture was spun at 313 x g for 5 minutes at RT, either to replace the medium or to dilute and maintain at 1-2 X 10^5 cells/ml. Cultures were maintained in a 75 cm^2 cell culture flasks with 2 µm vent caps (Corning) in a humidified incubator with 5% CO_2 at 37^{o}C. Cells were counted using an inverted microscope (Leica) and haemocytometer. THP-1 cell pellet was resuspended with medium and diluted 1:20 using 0.4% Trypan blue stain (made in PBS). 10 µl of this dilution was added to the haemocytometer and large, white, live cells were counted in the 4 (4 X 4) grids (dead cells stain blue) and cells/ml was calculated.

3.3.2 Freezing THP-1 cells

THP-1 cells were grown till they reached a cell count of 1 X 10^6 cells/ml (for example, 30 X 10^6 cells in 30 ml). Cells were counted as previously described and pelleted at 313 x g for 5 minutes at RT. The pellet was resuspended with THP-1 freezing solution: 1:10 dilution of sterile dimethyl sulfoxide (DMSO: Sigma) in sterile, heat inactivated FBS (to get final concentrations of 10% and 90% respectively), such that cell count is 1 X 10^7 cells/ml. Cells were transferred to cryovials (Thermo Scientific) as 500 µl aliquots of 1 X 10^7 cells/ml and frozen at -80^{o}C.

3.3.3 Thawing THP-1 cells

Frozen vials of THP-1 cells were thawed for 1-2 minutes in a 37^{o}C water bath. THP-1 maintenance medium was added dropwise to the cryovials. Once maximum volume was reached, the cells were transferred to a 15 ml tube and another 10 ml of THP-1 medium was added in a dropwise manner. The cells were pelleted at 313 x g for 5 minutes at RT. The pelleted cells were washed with 10 ml of THP-1 medium once more, resuspended in 5 ml of medium and maintained in a 25 cm^2 cell culture flask with 2 µm vent caps as previously described.

3.4 Identifying PfEMP1 domains of interest, selection of patient isolates expressing PfEMP1 domains of interest and primer design

PfEMP1 domains of interest, transcripts of which were upregulated in severe or uncomplicated malaria cases in individuals from Papua, Indonesia were identified by Dr. Michael Duffy and his group. The RNAseq and bioinformatics analyses carried out have been described in detail in [141] and have been described briefly section 6.2.3 of chapter 6. The details for selecting patient isolates expressing the PfEMP1 domains of interest and designing primers for amplifying the domains have been described in section 6.2.4 of chapter 6. The subsequent molecular biology methods for cloning the domains into expression vectors and transforming them into *E. coli* have been described in the following sections. In total, 22 PfEMP1 domains whose transcription was upregulated in severe malaria and 10 domains whose transcripts were up in uncomplicated malaria were successfully expressed. Details of the domains cloned and expressed can be found in table 6.4 and 6.5 of chapter 6.

3.5 Bacterial culture

3.5.1 Maintenance

Recombinant *Escherichia coli* (XL-10-Gold® strain) colonies (carrying the recombinant domains of interest) were grown on sterile Luria Bertini (LB) plates (1% w/v tryptone, 0.5% w/v yeast extract, 1% w/v NaCl, 1.5% w/v agar) with sterile 100 µg/ml ampicillin (sodium ampicillin, Sigma) at 37^{o}C and maintained at 4^{o}C for a maximum of four weeks.

3.5.2 Making ultra-competent *E. coli* (XL-10-Gold) cells

Freshly grown *E. coli* XL-10-Gold cells were used to make a pre-inoculum in sterile SOB medium (2% w/v tryptone, 0.5% w/v yeast extract, 0.05% w/v NaCl, 2.5 mM potassium chloride, 10 mM magnesium chloride). The pre-inoculum was used for the final inoculation in SOB medium which was incubated O/N at 21^{o}C, 180 rpm (rotations per minute). The culture was grown till an optical density (OD) of 0.55 was measured using a spectrophotometer. Cells were harvested at 1300 x g in a Sorvall SL9 3000 rotor at 4^{o}C for 10 minutes. The pellet was gently resuspended in ice-cold Inoue transformation buffer (55 mM manganese chloride, 15 mM calcium chloride, 250 mM potassium chloride, 10 mM PIPES (Sigma Aldrich)). The cells were harvested at 1300 x g for 10 minutes at 4^{o}C and the pellet was resuspended with ice cold Inoue buffer and DMSO. 50 µl aliquots were snap frozen using liquid nitrogen and stored at -80^{o}C.

3.6 Cloning

3.6.1 Polymerase chain reaction (PCR) amplification of domains of interest

DNA sequences for the domains of our interest were amplified from genomic DNA (gDNA) of parasites from malaria infected individuals (S10 supplementary data from [141] and appendix I) by polymerase chain reaction (PCR) using KAPAHiFi polymerase (Roche). The reaction was carried out in three main steps which were repeated over 30 cycles. The first step was carried out at 94^{o}C to allow the double stranded DNA (template) to be denatured. Next, single stranded oligonucleotide primers (listed in appendix I) annealed to the complementary strands on the template at a temperature of 50-59^{o}C (annealing temperature). Finally, at 68^{o}C, primers were extended with the complementary sequence of the template strand using polymerase and deoxynucleoside triphosphates (dNTPs). Eppendorf Gradient system was used to optimize the annealing temperature for step two of the reaction. Domains of interest were amplified using the Eppendorf Gradient system and Applied Biosystems GeneAmp® PCR system 9700.

3.6.2 Agarose gel electrophoresis

Agarose gel electrophoresis was used to size separate the DNA fragments. 6X DNA loading dye (Sigma) was added to the DNA samples which were then loaded on 0.9% agarose gels made in TAE buffer (40 mM Tris Base, 2 mM ethylene diamine tetraacetic acid (EDTA) pH 8, 0.35% glacial acetic acid). DNA was stained by adding SYBR® (Invitrogen) to the agarose gels. Separated DNA fragments were compared to 2-log DNA ladder (New England Biolabs) which was added in parallel to the DNA samples. Gels were run at 100-120 volts for 30-40 minutes or until the DNA fragments were well separated and visualized using the Safe-Imager Blue-Light Transilluminator or the Syngene G: Box.

3.6.3 Purification of DNA of interest by agarose gel extraction

After the domains of interest have been PCR amplified, the DNA fragments were size separated using agarose gel electrophoresis as described above. The DNA for the domains of interest were then extracted from the gel using the QIAquick® Gel extraction kit (Qiagen) as per the manufacturer's instructions. Briefly, the DNA fragment of interest was excised from the gel and weighed in an Eppendorf tube. The gel was dissolved by adding three times the volume (as the gel) of buffer QG and incubating at 50^{o}C for 10 minutes. Equal volume (as the gel) of isopropanol was then added and the contents transferred to the QIAquick column. The column was spun for 1 minute at 16,100 x g at RT. The column was washed with 500 µl of buffer QG to remove any remaining agarose

from the column. The DNA was then washed with 750 µl of buffer PE and centrifuged for 1 minute. The column was further centrifuged for a minute to remove any residual buffer PE. DNA was eluted by adding 50 µl of MQ H_2O to the centre of the column and incubating at RT for 1 minute before centrifuging the column at maximum speed for another minute and collecting the eluted DNA in an Eppendorf tube. Concentration of the extracted DNA was determined using agarose gel electrophoresis or NanoDrop Spectrophotometer.

3.6.4 Restriction digestion

The gel-extracted (as described above) DNA of interest and the plasmid DNA (pEU-E01-His-TEV-MCS-N1: pEU wheat germ expression vector) were digested using restriction enzyme (RE) double digestion as per the manufacturer's instructions. Briefly, 1 µg of DNA was digested with restriction enzymes (XhoI and XmaI or BamHI and NotI) (New England BioLabs) at 37^{o}C for 60-90 minutes. The digested fragments were analysed using agarose gel electrophoresis.

3.6.5 Purification of restriction enzyme (RE) digested DNA of interest

The digested DNA fragments were further purified using the QIAquick® PCR Purification kit (Qiagen) as per the manufacturer's protocol. Briefly, 5 volumes of buffer PB was added to 1 volume of our digested DNA and transferred to the QIAquick column. The column was centrifuged for 1 minute at 16,100 x g at RT. It was then washed with 750 µl of buffer PE. The column was spun for another minute to remove any residual wash buffer and DNA was eluted using 30 µl MQ H_2O after a minute incubation at RT. Concentration of the purified DNA was determined either by agarose gel electrophoresis or NanoDrop Spectrophotometer.

3.6.6 Ligation

The DNA of interest (insert) was ligated with the plasmid: pEU wheat germ expression vector (vector) using T4 DNA ligase (New England BioLabs). Based on the amount of DNA available after digestion and purification, a vector to insert molar ratio in the range of 1:1 to 1:13 was used. 20 µl reactions were set up, containing 1 µl of the 10X ligase buffer, 1 µl of T4 DNA ligase, ~75 ng of vector and ~50-200 ng of insert. The reaction was incubated at 16^{o}C, O/N.

3.6.7 Transformation

The ligated products were transformed into ultra-competent *E. coli* XL-10-Gold cells using the heat shock method. 5 µl of the ligated product was incubated with 50 µl of the ultra-competent cells on ice for 30 minutes under sterile conditions. Transformation was facilitated through heat shock at 42^{o}C for 40 seconds in a water bath followed by incubation on ice for a further 5 minutes. Cells were recovered using 600 µl of SOC medium without ampicillin (Media Preparation Unit, Peter Doherty Institute for Infection and Immunity, University of Melbourne) at 37^{o}C for 1 hour at 180 rpm. 200 µl of the transformation reaction mixture was plated on LB agar plates with 100 µg/ml ampicillin and grown at 37^{o}C O/N.

Transformed colonies were screened for the presence of the recombinant domains of interest by PCR as described above, using Taq DNA Polymerase (New England BioLabs) and primers specific for the vector pEU-E01-His-TEV-MCS-N1, which flanks our DNA of interest (Appendix II). PCR products were confirmed using agarose gel electrophoresis.

3.6.8 Glycerol stocks

Cultures of *E. coli* having the recombinant domains of interest were grown O/N in LB broth having 100 µg/ml ampicillin on a shaker at 180 rpm at 37^{o}C. Cells were harvested at 6900 x g for 3 minutes at RT, resuspended with 200 µl sterile LB: glycerol solution (1:1 dilution, without ampicillin) and stored directly at -80^{o}C.

3.6.9 Purification of plasmid DNA

E. coli colonies positive by PCR for our domains of interest were inoculated in LB medium with 100 µg/ml ampicillin and grown at 37^{o}C, 180 rpm, O/N. Recombinant plasmids were purified using the QIAprep® Spin Miniprep Kit (Qiagen) as per the manufacturer's instruction. The bacterial cells were pelleted at 6900 x g, RT for 3 minutes. The pelleted cells were resuspended using 250 µl buffer P1 and lysed using 250 µl buffer P2. Contents were mixed thoroughly by inverting the tubes gently. 350 µl buffer N3 was then added, the contents mixed and spun at top speed (16,100 x g) for 10 minutes at RT. The supernatants were transferred to the QIAprep spin columns and centrifuged at top speed for 1 minute at RT. Washes were carried out with 500 µl buffer PB and 750 µl of buffer PE. The columns were further spun for 1 minute after the washes to remove any residual buffer. The columns were placed in an Eppendorf tube and 50 µl MQ H_2O was added. The columns were incubated at RT for 1 minute and plasmid DNA was eluted by

centrifuging at top speed for 1 minute. Concentration and quality of the purified plasmid was verified by agarose gel electrophoresis and NanoDrop Spectrophotometer.

Presence of our domains of interest in the purified plasmids was confirmed by PCR using Taq DNA Polymerase (New England BioLabs) and insert specific primers (Appendix I).

3.6.10 DNA sequencing

Sequences of our domains of interest were confirmed by sending our purified plasmids to Australian Genome Research Facility (AGRF, Walter and Eliza Hall Institute, Parkville). 1 pmol of sequencing primer (Appendix II) and 600-1200 ng of purified plasmid in a total volume of 12 µl was sent to the AGRF facility. The sequences were analysed using Geneious R10 (Biomatters, New Zealand). The sequences were confirmed by comparing with the DNA sequence of parasites infecting the selected patient (the selected patients were the 'targets' used for amplifying the domains of interest. Details of the patient isolates selected have been mentioned in the appendix I (targets), while the method of patient selection has been described in chapter 6, section 6.2.4). Further confirmation of the cloned domain sequences was carried out using the De novo assembly feature of the Geneious R10 software. Sequences with insertion or deletion mutations, gaps or mismatches were identified and not used further for protein expression.

3.7 Wheat germ cell free protein expression

Domains of interest successfully cloned in the pEU wheat germ expression vector (table 6.4) were shipped to Japan and expressed using Wheat Germ Cell Free Protein Synthesis Systems (WGCFS) (table 6.5) by Professor Takafumi Tsuboi and Dr. Eizo Takashima (Ehime University, Matsuyama, Japan) and CFS Co., Ltd, Matsuyama, Japan as previously described [316, 317]. Briefly, the cloned domains of interest were transcribed, and the mRNA translated using bilayer or discontinuous batch translation method using the wheat germ cell free machinery. This yielded ~ 11-200 µg of purified proteins with an N-terminal 6-Histidine (HIS) tag.

3.8 Antibody measurement assays

3.8.1 Multiplex assays

3.8.1.1 MAGPIX multiplex assay

3.8.1.1.1 Carbodiimide coupling of protein to Bio-Plex magnetic carboxylated microspheres

Protein coupling was carried out as previously described [318, 319] with a few modifications. Bio-Plex magnetic carboxylated microspheres (Bio-Rad, 1.25 X 10^7 beads/ml) were resuspended by vortexing and sonication for 30 seconds. All washes were carried out using the Bio-Rad magnetic separator and pipetting out the supernatant. 100 µl of each bead region (1.25 X 10^6 beads/ml) were activated using 80 µl sterile activation buffer (0.1 M NaH_2PO_4, pH 6.2). 10 µl each of 50 mg/ml Sulfo-NHS (N-hydroxysulfosuccinimide, Thermo Fisher Scientific, 2 mg) and 50 mg/ml of Pierce™ Premium Grade EDC (Thermo Fisher Scientific) was freshly made and added to the beads. The beads were incubated in the dark for 30 minutes at RT on a rotator set at maximum speed. Beads were washed twice with 150 µl sterile coupling buffer (0.1 M MES hemisodium salt in MQ.H_2O, pH 6.1) (Sigma-Aldrich). 5-12 µg of the protein to be coupled along with required amount of coupling buffer was added to the beads, vortexed gently and incubated for 3 hours in dark on the rotator at RT, maximum speed. The beads were washed with sterile 1X PBS and incubated with sterile blocking buffer (0.1% BSA, 0.02% Tween 20, 0.05% sodium azide in 1X PBS) for 30 minutes, RT rotator. The beads were first washed with sterile storage buffer (0.05% sodium azide in 1X PBS) and resuspended and stored the beads in the same storage buffer. Bead count was confirmed using the haemocytometer. Beads coupled to proteins were stored at 4°C for up to one month or at -80°C for 6 months. Although the level of protein coupling to the beads was not assessed directly, the pooled plasma from malaria infected individuals, positive for total IgG levels (PPS, heat inactivated or non-heat inactivated) showed higher antibody binding/ recognition of the protein coupled beads as compared to test plasma samples. This was used as a positive indication of protein successfully coupling to the beads.

3.8.1.1.2 Antibody measurement using MAGPIX multiplex assay

Antibodies were measured against the antigens coupled to the microspheres as previously described [318, 319]. Briefly, a working bead mixture of the protein-coupled beads was prepared by diluting the different protein-beads stock using sterile assay buffer (1% BSA in 1X PBS) to get a final concentration of 10 beads/ µl for 1 protein-bead region (500 beads/well in 50 µl). 50 µl of the bead mixture was added to a 96-well flat-

bottom black plate (Bio-Plex Pro™, Bio-Rad). 50 µl of non-heat inactivated patient plasma (diluted 1:50 in PBS) were added to the wells. Non-heat inactivated serum from Melbourne naïve individuals and a no-plasma control were used as the negative controls. Two sets of pooled plasma from malaria infected individuals, positive for total IgG levels (PPS) were used as positive controls, while a 5-fold, nine-point serial dilution of total IgG (Sigma-Aldrich), starting with 500 µg/ml concentration was used for the standards. The plates were incubated O/N at 4°C on a plate shaker set to medium speed in the dark. The plates were spun at 2885 x g, for 3 minutes at RT. The plates were washed with wash buffer (0.1% Tween in 1X PBS) using the BioPlex Pro II wash station. 50 µl of 1.3 µg/ml detector antibody (mouse anti-human IgG-PE, Southern Biotech) diluted in assay buffer was added and incubated for 2 hours on a RT plate shaker. The plates were washed twice with Magpix sheath fluid and further incubated in the sheath fluid for 10 minutes on the RT plate shaker.

The median fluorescence intensity (MFI) was measured using the MAGPIX machine (Bio-Plex® MAGPIX multiplex reader, Bio-Plex Manager, Bio-Rad). Average MFI levels for the no-plasma control was considered as background signal and subtracted from the MFI values of each test sample. Plate variation was normalized using the MFI values measured against each antigen for the standards, the Melbourne naïve controls and pooled positive plasma controls, which were the common controls used in all plates.

3.8.1.2 Antibody measurement using Luminex multiplex assay

This assay was carried out in the laboratory of Associate Professor Thomas Lavstsen at the Centre of Medical Parasitology, University of Copenhagen using protein-coupled beads made by his group. Antibody levels were measured against recombinant CIDR domains using this assay as described by Cham and group [320]. The HIS-tagged domains were expressed using the Drosophila Sf9 cells and purified by nickel affinity chromatography and coupled to carboxylated luminex beads. Details of the domains used can be found in Supplementary table 2 of chapter 4, and details of their production and amino acid sequence have been referenced in [128, 130, 229]. Plasma samples diluted 1:160 in Assay Buffer E (ABE: 0.1% BSA, 0.05% Tween-20 in PBS, pH 7.4) were used. Using the same buffer, a ten point, two-fold dilution of pooled positive plasma (PPS), positive for malaria infection was carried out starting with a 1:40 dilution. Malaria naïve Danish individuals were used as negative controls. A working mixture of the protein-coupled beads (starting concentration 1.25 X 10^7 beads/ml) was prepared by first sonicating and then mixing equal volumes of each domain of interest and diluting them 1:333 in ABE. 50 µl of the beads working stock was mixed with 50 µl of patient plasma in

a 96-well microtiter plate (MSBVS 1210, Millipore, USA) pre-wetted with ABE. The plates were incubated at RT on a plate shaker for 30 minutes in the dark. Plate washes were carried out with ABE using a vacuum-assisted plate washer. 50 µl of phycoerythrin-conjugated Goat Anti-human IgG (Jackson ImmunoResearch Laboratories), diluted 1:3500 was added and incubated for 30 minutes on the RT shaker. The plates were washed and stored in ABE, O/N at 4^{o}C. Next day, the plates were washed and resuspended with ABE using the RT shaker.

Using the BioPlex^{100} system, mean fluorescent intensities (MFI) were measured. A standard curve was generated using the PPS, and an arbitrary value of 1000 Relative Units (RU) was assigned to the highest concentration. IgG concentrations for the test samples were then interpolated from these standard curves.

3.8.2 Opsonic phagocytosis assay

Opsonic phagocytosis of IE was measured by a flow cytometry-based assay as previously described [265]. IE with 5-8% trophozoites were first purified using the Percoll gradient method as described above. The purified trophozoites were stained with Dehydro Ethidium Bromide (DHE: 10 mg/ml in DMSO) (Sigma) in a 1:400 dilution for 30 minutes. The stained IE were washed thrice with RPMI-HEPES wash buffer at 350 x g for 3 minutes at RT to remove the excess stain. 96-well u-bottom plates (Falcon, Corning) coated with 0.1% casein (made in PBS) were used for the assay. Heat inactivated patient plasma were incubated with 1.65 X 10^7 cells/ml of DHE stained IE (for opsonization) to get a final dilution of 1:10, for 1 hour at RT, under dark conditions. The opsonized cells were washed thrice, resuspended in 50 µl of THP-1 maintenance medium and aliquoted into duplicates in new casein coated plates with 25 µl volume of each duplicate. Phagocytosis was carried out by incubation with undifferentiated THP-1 cells at a concentration of 5 X 10^5 cells/ml, for 40 minutes in a 5% CO_2 humidified incubator at 37^{o}C. For each duplicate in each well, the THP-1: IE ratio was 1:10 (2.5 X 10^4 THP-1 cells: 2.5 X 10^5 IE). Phagocytosis was stopped by spinning the plates at 350 x g for 3 minutes at 4^{o}C. The non-phagocytosed IE and the uninfected RBCs were lysed using the FACS lysing solution (BD Biosciences) made by diluting 1:10 in sterile MQ H_2O. The cells were washed with FACS buffer (2% FBS made in sterile PBS) 2-3 times, fixed with 120 µl of 2% chilled paraformaldehyde (made in PBS) and incubated O/N at 4^{o}C. For opsonic phagocytosis measurement of the rosetting parasite line, 100 µg/ml heparin was added to the RPMI-HEPES wash buffer and the THP-1 maintenance medium to disrupt the rosettes.

Cells were acquired using the Cyan, HyperCyt flow cytometer or CytoFLEX S (Beckman Coulter) for 30 seconds with ~500 events/second. THP-1 cells were gated based on the forward and side scatter plots and gates were set such that 5% of the THP-1 cells from the negative control (no serum/plasma control: has unopsonised IE with THP-1 cells only) are DHE positive. Percent phagocytosis (the proportion of THP-1 cells that phagocytosed the IE) was calculated, first by subtracting the average DHE positive THP-1 cells in the no serum negative control from the average DHE positive THP-1 cells in the test sample and the positive control (rabbit anti-human IgG against human RBCs from Cappel, MP Biomedicals). The percent phagocytosis was then calculated relative to the positive control. Duplicate samples showing more than 20% variance in their % phagocytosis levels were repeated.

3.9 Statistical analyses

Statistical analyses were carried out using STATA13.0 (StataCorp LP, Texas, USA) software, GraphPad PRISM5 (La Jolla, CA, USA) software and MATLAB version 2017b with the Statistics and Machine Learning Toolbox (MathWorks, Massachusetts, USA). Details of the analyses carried out are described in the statistical analyses section of each results chapter.

4 Chapter 4: Acquisition of antibodies against endothelial protein C receptor-binding domains of *Plasmodium falciparum* erythrocyte membrane protein 1 in children with severe malaria

4.1 Introduction

The current chapter determined acquisition of antibodies against a panel of CIDR domains of PfEMP1 in PNG children with severe or uncomplicated malaria. Total IgG levels were measured against 35 recombinant CIDR domains in plasma collected from PNG children at hospital presentation (acute) and in convalescence following severe or uncomplicated malaria, and in age-matched community controls. The study also investigated if antibodies against the CIDR domains varied between disease presentation and convalescence, two months after the malaria episode. The 35 recombinant CIDR domains were grouped based on their binding phenotype into those that bind EPCR, subdivided into EPCR binders belonging to group A *var* genes and EPCR binders belonging to group B/A *var* genes; CIDR domains binding CD36 and those binding neither EPCR or CD36 (possibly associated with rosetting). Findings for this chapter were reported in a first author publication presented in the next few pages.

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MAJOR ARTICLE

Acquisition of Antibodies Against Endothelial Protein C Receptor–Binding Domains of *Plasmodium falciparum* Erythrocyte Membrane Protein 1 in Children with Severe Malaria

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Background. *Plasmodium falciparum* erythrocyte membrane protein 1 (PfEMP1) mediates parasite sequestration in postcapillary venules in *P. falciparum* malaria. PfEMP1 types can be classified based on their cysteine-rich interdomain region (CIDR) domains. Antibodies to different PfEMP1 types develop gradually after repeated infections as children age, and antibodies to specific CIDR types may confer protection.

Methods. Levels of immunoglobulin G to 35 recombinant CIDR domains were measured by means of Luminex assay in acute-stage (baseline) and convalescent-stage plasma samples from Papua New Guinean children with severe or uncomplicated malaria and in healthy age-matched community controls.

Results. At baseline, antibody levels were similar across the 3 groups. After infection, children with severe malaria had higher antibody levels than those with uncomplicated malaria against the endothelial protein C receptor (EPCR) binding CIDRα1 domains, and this difference was largely confined to older children. Antibodies to EPCR-binding domains increased from presentation to follow-up in severe malaria, but not in uncomplicated malaria.

Conclusions. The acquisition of antibodies against EPCR-binding CIDRα1 domains of PfEMP1 after a severe malaria episode suggest that EPCR-binding PfEMP1 may have a role in the pathogenesis of severe malaria in Papua New Guinea.

Keywords. PfEMP1; *Plasmodium falciparum*; severe malaria; antibodies; Papua New Guinea; Luminex assay; CIDR; EPCR.

Malaria caused 429 000 deaths in 2015, deaths due mainly to *Plasmodium falciparum* infection in young children [1]. *P. falciparum*–infected erythrocytes (IEs) avoid splenic clearance through sequestration in the deep vasculature, leading to organ-specific damage and development of complications, such as cerebral [2–4] or placental malaria [5]. Sequestration is mediated by the *P. falciparum* erythrocyte membrane protein 1 (PfEMP1) family of clonally variant, multidomain adhesins, which are exported to the IE surface and exposed to host antibodies [6]. Encoded by a repertoire of about 60 *var* genes per parasite genome, PfEMP1 types mediate adhesion to host receptors, including intercellular adhesion molecule 1 [7], CD36 [8], complement receptor 1 [9], chondroitin sulfate A [10], and endothelial protein C receptor (EPCR) [11].

Receptor availability and host immune status influence the PfEMP1 phenotype of the infecting parasites [12]. Severe malaria is associated with widespread organ sequestration [13, 14] and is thought to be caused by parasites expressing a specific subset of PfEMP1 binding to and functionally impairing EPCR [11], a key endothelial receptor with cytoprotective, anticoagulative, and anti-inflammatory effects [15].

PfEMP1 consists of multiple Duffy binding–like domains, and cysteine-rich interdomain regions (CIDRs), which can be classified as CIDR α, β, γ, and δ [16, 17]. PfEMP1 containing CIDRα1 domains and CIDRα2–6 domains bind to EPCR [18] and CD36 [19] respectively, whereas PfEMP1 with CIDR β, γ, or δ domains are possibly associated with rosetting [20]. *Var* genes are classified into groups A, B, and C based on their upstream promoter sequences, transcriptional direction, and

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chromosomal location [21]. Domain cassettes (DCs) are conserved sets of PfEMP1 domains that occur together, with the chimeric group B/A PfEMP1 type belonging to DC8 [16]. Group A [12, 22] and group B/A [23] *var* genes have been associated with severe malaria in African children.

Naturally acquired antibodies may confer protective immunity against malaria (reviewed in [24]), and immunity to severe malaria may be acquired after 1 or 2 severe infections [25]. Antibodies to IEs mainly target PfEMP1 [26], and the PfEMP1 antibody repertoire increases with repeated exposure to malaria infections [27]. Antibodies against group A PfEMP1 types tend to be acquired before those against group B and C [28], and antibodies that can block the binding of CIDRα1 domains to EPCR are acquired early in life in areas of high malaria transmission [29].

Studies of *var* gene expression have shown parasites expressing EPCR-binding PfEMP1 to be associated with severe malaria in African children [12, 22, 23, 30] and Indian adults [31]. Fewer data exist for Melanesia, where severe malaria case fatality rates are relatively low, and where *Plasmodium vivax* and mixed infections also commonly cause severe malaria [32].

In Papua New Guinea (PNG), children with severe malaria had high levels of transcription of group A *var* genes [33] and genes encoding EPCR-binding [34] or rosetting PfEMP1 types [35]. Group B *var* transcription was higher than groups A or C *var* transcripts in children with severe or uncomplicated malaria, and group C transcription was elevated in asymptomatic children [35]. Recently, RNA sequencing analysis of patient isolates corroborated the notion that parasites expressing EPCR-binding PfEMP1 are associated with severe disease in Timika, Indonesia [36].

Antibodies against PfEMP1 in PNG increased with age and were associated with reduced risk of symptomatic malaria [37], whereas antibodies from individuals with uncomplicated malaria recognized group C PfEMP1 types [34]. Complementary research conducted with the same severe malaria study population found that antibodies to the IE surface predominantly targeted PfEMP1. Furthermore, levels of antibodies to PfEMP1 expressed on IEs (DC8 and DC13 variants) were higher in uncomplicated malaria than severe malaria, suggesting these antibodies could protect from severe malaria [38]. To investigate the role of antibody acquisition to specific PfEMP1 domains in immunity against severe malaria in PNG, we compared levels of antibodies against a broad panel of CIDR domains in young children with severe or uncomplicated malaria and matched healthy controls.

MATERIALS AND METHODS

Study Population

Study participants were children aged 0.5–10 years presenting to Modilon Hospital, Madang, with severe or uncomplicated *P. falciparum* malaria, together with community-recruited age-matched children [39]. Severe malaria was defined based on World Health Organization criteria [40], including a Blantyre coma score (BCS) <5 or impaired consciousness, severe anemia (hemoglobin, <50 g/L), respiratory distress, prostration, multiple seizures, hyperlactatemia (blood lactate, >5 mmol/L); dark urine, hypoglycemia (blood glucose, ≤2.2 mmol/L), jaundice, abnormal bleeding, persistent vomiting, or signs of shock [39]. Key severe malaria presentations are summarized in Supplementary Table 1; children with uncomplicated malaria had no criteria for severe malaria. Healthy asymptomatic children from the Madang region were recruited as community controls. Controls were matched by age (±12 months), sex, and ethnicity. Spleens were palpable in 13%; the prevalence of *P. falciparum* was 8.2% and the prevalence of *P. vivax* 14.1% by microscopy. *P. falciparum* infection was confirmed with microscopy, and speciation with nested polymerase chain reaction [39].

At presentation, venous blood was collected and plasma was separated by centrifugation and stored at −70°C, until shipping to Melbourne. In children with malaria, convalescent plasma was similarly collected 8 weeks later.

Samples from 112 children per group were selected. For the final analysis, some children were excluded. Five children with severe malaria lacked complete clinical information, 1 experiment failed, and 8 had *P. vivax* infection. Four children with uncomplicated malaria lacked complete clinical information, 19 had *P. vivax* infection, 7 did not have parasite densities recorded, and 17 did not have polymerase chain reaction– or microscopy-confirmed *P. falciparum* infection. Thus, results are presented for 98 children with severe malaria, 65 with uncomplicated malaria, and 112 community controls (Table 1).

Ethics

Written informed consent was obtained from the child's parent or guardian. Ethical approval was given by the PNG Institute of Medical Research Institutional Review Board (IRB No. 1103) and the Medical Research Advisory Committee of the PNG Health Department (MRAC No. 11.12). The study was conducted according to the principles of the Declaration of Helsinki [39].

Luminex Assay and Measurement of Immunoglobulin G Levels

A total of 35 recombinant HIS-tagged CIDR domains (Supplementary Table 2) were expressed in Drosophila Sf9 cells and purified by nickel affinity chromatography, as described elsewhere [18, 29]. Total immunoglobulin G (IgG) levels against these proteins were measured using a published Luminex assay [41], with minor modifications. Briefly, plasma samples were diluted 1:160 in assay buffer E (ABE; 0.1% bovine serum albumin, 0.05% Tween-20 in phosphate-buffered saline; pH7.4). In ABE, a 10-point, 2-fold dilution of pooled positive plasma

Table 1. Summary of Study Population Categorized by Disease Severity

Characteristic	Severe Malaria (n = 98)	Uncomplicated Malaria (n = 65)	Community Controls (n = 112)[a]
Age, median (IQR), mo	42 (30–60)	45 (34–59)	42 (30–58)
Age group, %			
0–48 mo	61.2	53.9	57.7
49–130 mo	38.8	46.1	42.3
Sex, %			…
Female	45.9	41.5	N/A
Male	54.1	58.5	N/A
Ethnicity, %			
Madang	78.6	89.2	79.5
Madang Sepik	7.1	4.6	9.8
Other	5.1	6.2	0
Sepik	9.2		10.7
Hemoglobin, median (IQR), g/L	79 (55–91)	89 (72–100)	104 (92–113)
Initial *Plasmodium falciparum* density, median (IQR), parasites/μL	86 171.5 (27 064–18 1072)	21938 (4811–66 053)	…

Abbreviation: IQR, interquartile range; N/A, not available.

[a]For age, age group, and hemoglobin variables, n = 111.

starting with a dilution of 1:40 was carried out. Equal volumes of beads (initial microsphere concentration, 1.25×10^7/mL) coupled to each domain of interest were mixed and then diluted 1:333 in ABE. Next, 50 μL of beads and 50 μL of diluted plasma were added to 96-well microtiter plates (MSBVS 1210; Millipore) prewetted with ABE. After that, 50 μL of phycoerythrin-conjugated goat anti-human IgG (Jackson ImmunoResearch Laboratories), diluted 1:3500, was added, and the Bio Plex 100 system (Bio-Rad) was used to measure mean fluorescent intensities and antibody concentrations. Using the pooled positive plasma, a standard curve was generated with an arbitrary value of 1000 relative units assigned to the highest concentration, and IgG concentrations were interpolated from the curves.

Statistical Analysis

The 35 recombinant CIDR domains were grouped (Supplementary Table 2) into those binding EPCR [18] (CIDRα1.1 and CIDRα1.4–1.8; n = 19), subdivided into groups A (CIDRα1.4–1.7; n = 13) and B/A (CIDRα1.1 and CIDRα1.8; n = 6); those binding CD36 [19] (CIDRα2–6; n = 12); and those binding neither EPCR nor CD36 [29] (CIDRδ [n = 3] and CIDRγ [n = 1]). Mean antibody levels to these proteins were compared among and within the different sample groups (severe and uncomplicated acute stage, severe and uncomplicated convalescent stage, and community controls), across different age groups and against each of the CIDR domain types.

Stata 13 (StataCorp) and GraphPad Prism 5 software were used for statistical analysis. Wilcoxon rank sum tests or Kruskal-Wallis tests were performed to compare antibody levels in paired groups or multiple groups, respectively. To determine the relationship between age and antibodies to CIDR domains, participants were divided into younger children (0–48 months old) and older children (49–130 months old). To determine changes in antibody levels between baseline and follow-up, a Wilcoxon matched-pairs signed rank test was carried out.

RESULTS

A total of 438 plasma samples were analyzed from 98 children with severe *P. falciparum* malaria, 65 with uncomplicated *P. falciparum* malaria, and 112 age-matched controls (Table 1).

Antibody Levels at Presentation in Severe Malaria and Uncomplicated Malaria

At presentation, antibody levels against the CIDR domains were similar between children with severe malaria, children with uncomplicated malaria, and community controls (Figure 1). The exception was for antibody to CD36-binding CIDR domains, which was higher in those with uncomplicated malaria than in community controls ($P = .01$) (Figure 1).

When antibody levels against individual domains were compared, children with severe malaria had higher levels of antibody against CIDRα1.5b(b) than those with uncomplicated malaria. Children with uncomplicated malaria had higher levels of antibody against CIDRα1.8b(a), CIDRα3.3, and CIDRγ3 domain than children with severe malaria (Supplementary Table 3).

Association of Age With Antibody Levels at Baseline

To investigate whether age was a major determinant of antibodies to CIDR domains, we compared antibody levels at presentation by disease severity in younger and older children separately. In younger children, no differences were observed between severe and uncomplicated malaria for any CIDR types, but community controls had lower levels of antibodies to most CIDR types than children with severe or uncomplicated malaria (Figure 2). In older children, antibody levels were similar at study entry in all 3 groups (Figure 2). These data probably

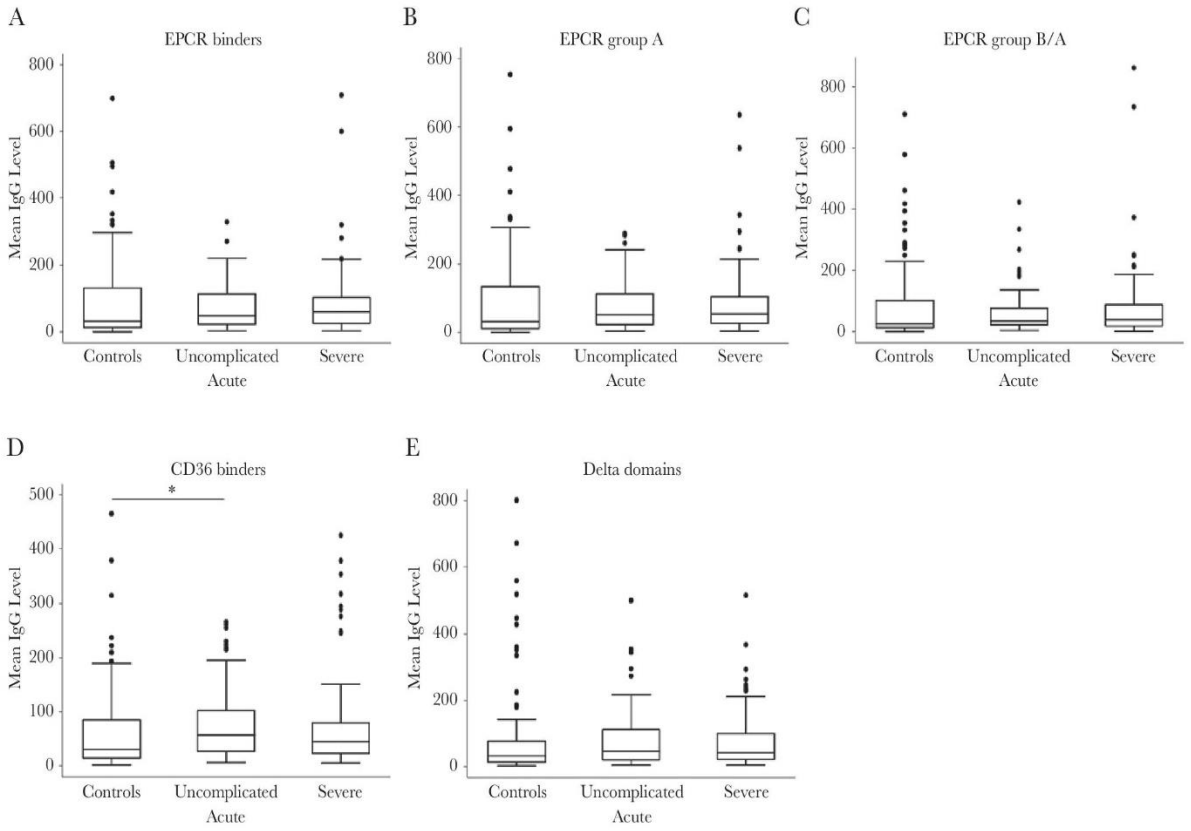

Figure 1. Levels of immunoglobulin G (IgG) against different cysteine-rich interdomain region (CIDR) domain types in participants belonging to different disease severity groups at baseline (acute stage). Kruskal-Wallis tests were used to compare community controls (n = 112) and participants with uncomplicated (n = 65) or severe (n = 98) acute malaria. Values on the y-axes represent mean IgG levels in relative units; box plots show medians with interquartile range and outliers. Mean levels of IgG against the following CIDR domain types were measured: binding endothelial protein C receptor (EPCR) (*A*); binding EPCR, group A (*B*); binding EPCR, group B/A (*C*); binding cluster of differentiation 36 (CD36) (*D*); and delta domains associated with rosetting (*E*). *P = .01 (Wilcoxon rank sum test).

reflect lower exposure to malaria among the younger children, a notion also supported by the higher antibody levels observed in older compared to younger community controls against all the CIDR domain types (Supplementary Figure 1).

Antibody Levels in Convalescence After Severe or Uncomplicated Malaria

In convalescent plasma, mean levels of antibody against EPCR-binding CIDR domains were higher in children with severe malaria than in community controls (Figure 3A), and levels of antibody against group B/A EPCR-binding CIDR domain proteins were higher in severe than in uncomplicated malaria (Figure 3C). No other CIDR antibody levels varied between groups (Figure 3).

Convalescent antibody levels against 7 individual CIDRα1 domains and also against CIDRα2.9 were significantly higher in children with severe malaria than in those with uncomplicated malaria. These variants included multiple CIDRα1.1, CIDRα1.5, and CIDRα1.8 domains and a CIDRα1.7 domain (Supplementary Table 4). Levels of antibodies against other non–EPCR-binding domains did not vary between severe and uncomplicated malaria.

Associations Between Age and Convalescent Antibody Levels

When convalescent plasma antibody levels were stratified by age, among older children, those with severe malaria had significantly higher levels of antibody against EPCR-binding CIDRs than those who recovered from uncomplicated malaria (Figure 4A, 4B, and 4C). No such differences were observed against CD36-binding CIDRs or CIDRδ domain types. In younger children, the antibody levels did not differ significantly by malaria severity for any of the CIDR domain types tested (Figure 4).

In older children, antibody levels in convalescent plasma were similar in community controls and severe or uncomplicated malaria, but in younger children the community controls had lower antibody levels against most CIDR domain types than children convalescent from either type of malaria (Figure 4).

Comparing the antibody levels between younger and older children after severe malaria or uncomplicated malaria separately, we found higher antibody levels to EPCR-binding CIDRs in older children than in younger children after severe malaria. Such differences in antibody levels were not observed in children

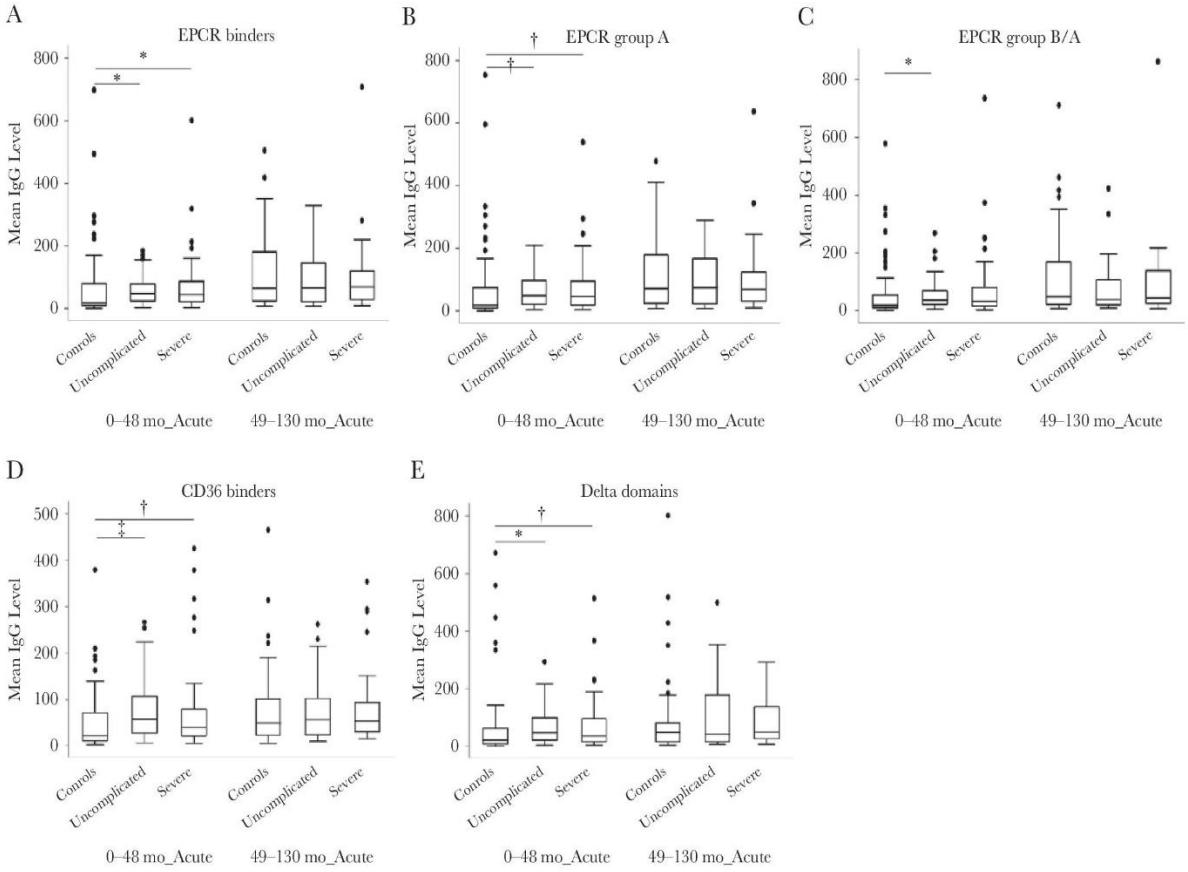

Figure 2. Levels of immunoglobulin G (IgG) against different cysteine-rich interdomain region (CIDR) domain types in participants belonging to different disease severity groups and grouped according to age at baseline (acute stage). Wilcoxon rank sum tests were used to compare community controls and participants with uncomplicated or severe acute-stage malaria group in the 0–48-month age group; similar comparisons were done for participants in the 49–130-month age group. Values on the y-axes represent mean IgG levels in relative units; box plots show medians with interquartile range and outliers. Total number of participants in community control, uncomplicated acute malaria, and severe acute malaria groups: n = 64, 35, and 60, respectively, in the 0–48-month age group, and n = 47, 30, and 38, respectively, in the 49–130-month age group. Mean levels of IgG against the following CIDR domain types were measured: binding endothelial protein C receptor (EPCR) (*A*); binding EPCR, group A (*B*); binding EPCR, group B/A (*C*); binding cluster of differentiation 36 (CD36) (*D*); and delta domains associated with rosetting (*E*). *P = .02; †P = .03; ‡P = .002.

convalescent from uncomplicated malaria (Supplementary Figure 1).

Temporal Changes in Antibody Levels Against Different CIDR Types

We determined whether antibody levels against different CIDR types changed between presentation and convalescence. For severe malaria, antibody levels against EPCR-binding CIDRα1 domains belonging to group A were higher in convalescent-stage than in acute-stage plasma samples (hereafter "convalescent" and "acute"). No differences were observed for the other CIDR domain types (Table 2). When stratifying by age, increases in antibody to CIDRα1 domains were restricted to older children (Table 3).

Children with uncomplicated malaria showed a significant decrease in antibody levels against group B/A CIDR EPCR binders (for the other CIDR domain types this decrease was nonsignificant) (Supplementary Table 5). In older children with uncomplicated malaria, antibody levels against EPCR-binding CIDR domains decreased significantly during follow-up (except for group B/A CIDR EPCR binders, for which younger children showed a decrease in antibody levels; P = .03) (Supplementary Table 6).

Antibody Levels in Children With Different Severe Malaria Syndromes

We investigated whether acute or convalescent antibody levels against CIDR domains were associated with specific severe malaria syndromes. We separately compared children with or without severe anemia, respiratory distress, metabolic acidosis, and hyperlactatemia and children with different BCS scores (Supplementary Table 1). Children with overlapping severe malaria syndromes are represented in Supplementary Figure 2.

In acute plasma, antibody levels did not differ significantly between severe malaria syndromes. In convalescence, children with metabolic acidosis had higher levels of antibody against CIDRδ domains than those without metabolic acidosis, and this difference was significant among younger children. Antibody levels against other domain types did not differ significantly (Supplementary Figure 3).

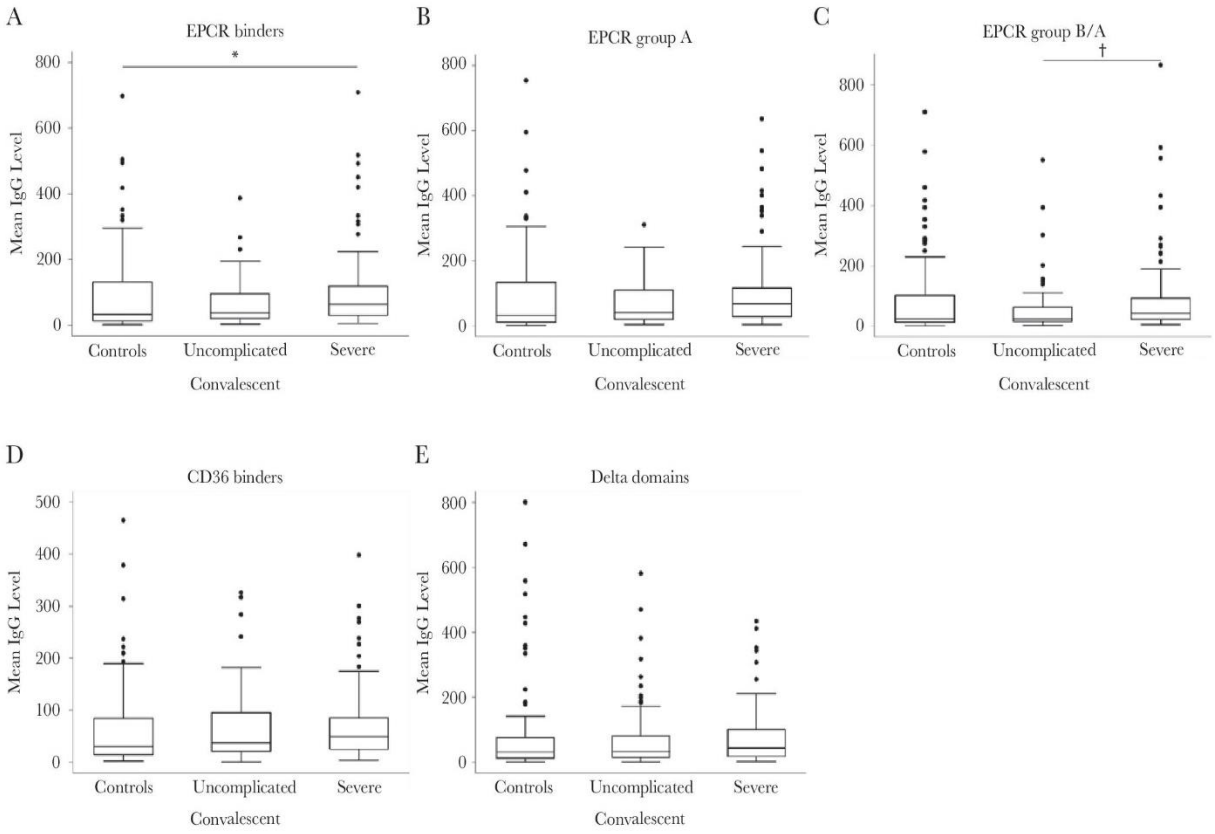

Figure 3. Levels of immunoglobulin G (IgG) against different cysteine-rich interdomain region (CIDR) domain types in convalescent-stage plasma samples from children in different disease severity groups. Kruskal-Wallis tests were used to compare community controls (n = 112) and uncomplicated (n = 65) and severe (n = 98) malaria groups (convalescent stage). Values on the y-axes represent mean IgG levels in relative units; box plots show medians with interquartile range and outliers. Mean levels of IgG against the following CIDR domain types were measured: binding endothelial protein C receptor (EPCR) (*A*); binding EPCR, group A (*B*); binding EPCR, group B/A (*C*); binding cluster of differentiation 36 (CD36) (*D*); and delta domains associated with rosetting (*E*). **P* = .03; †*P* = .01 (both Wilcoxon rank sum test).

In convalescent plasma, children with impaired consciousness (BCS score, 0–4) had higher levels of antibody against group B/A CIDR EPCR binders than children with uncomplicated malaria (Supplementary Figure 4). No differences in antibody levels were observed when comparing children with cerebral malaria (BCS score ≤ 2) with children with impaired consciousness (BCS score, 3 or 4) or with a BCS score 5.

DISCUSSION

PfEMP1 molecules can be grouped into mutually exclusive binding phenotypes determined by their N-terminal CIDR domains. These mediate adhesion to EPCR [11] or CD36 [19] or have unknown functions possibly associated with rosetting [20]. Using acute and convalescent plasma from children from PNG, we found malaria syndrome-specific and age-dependent differences in antibody recognition of different types of CIDR domains.

At baseline, similar levels of antibody against CIDR domains were observed across all subject groups. Age significantly affected antibody levels: younger community controls had lower antibody levels against most CIDR domain types than matched children with severe or uncomplicated malaria or older community controls, possibly reflecting their lack of exposure to malaria [42]. The antibody reactivity detected at presentation could result from either previous or ongoing infections.

In convalescence, children with severe malaria had higher levels of antibody against EPCR-binding CIDRα1 domains than the community controls, and higher levels than the uncomplicated malaria group for the group B/A (DC8) CIDRα1 domain types. With results stratified by age, older children with severe malaria had higher levels of antibody to EPCR-binding CIDRα1 domains belonging to group A or B/A and to several individual CIDRα1 domain variants than children with uncomplicated malaria. Comparing acute and convalescent antibody levels in children with severe malaria, we found increases in antibody levels against the group of EPCR-binding CIDRα1 domains, belonging to group A and B/A PfEMP1 types. This association was restricted to older children. Overall, differences in antibody responses observed after stratification by age are in line with findings of a recent Ugandan study demonstrating strong associations between increasing age and antiparasite (density) and antidisease (parasite density with fever) immunity after

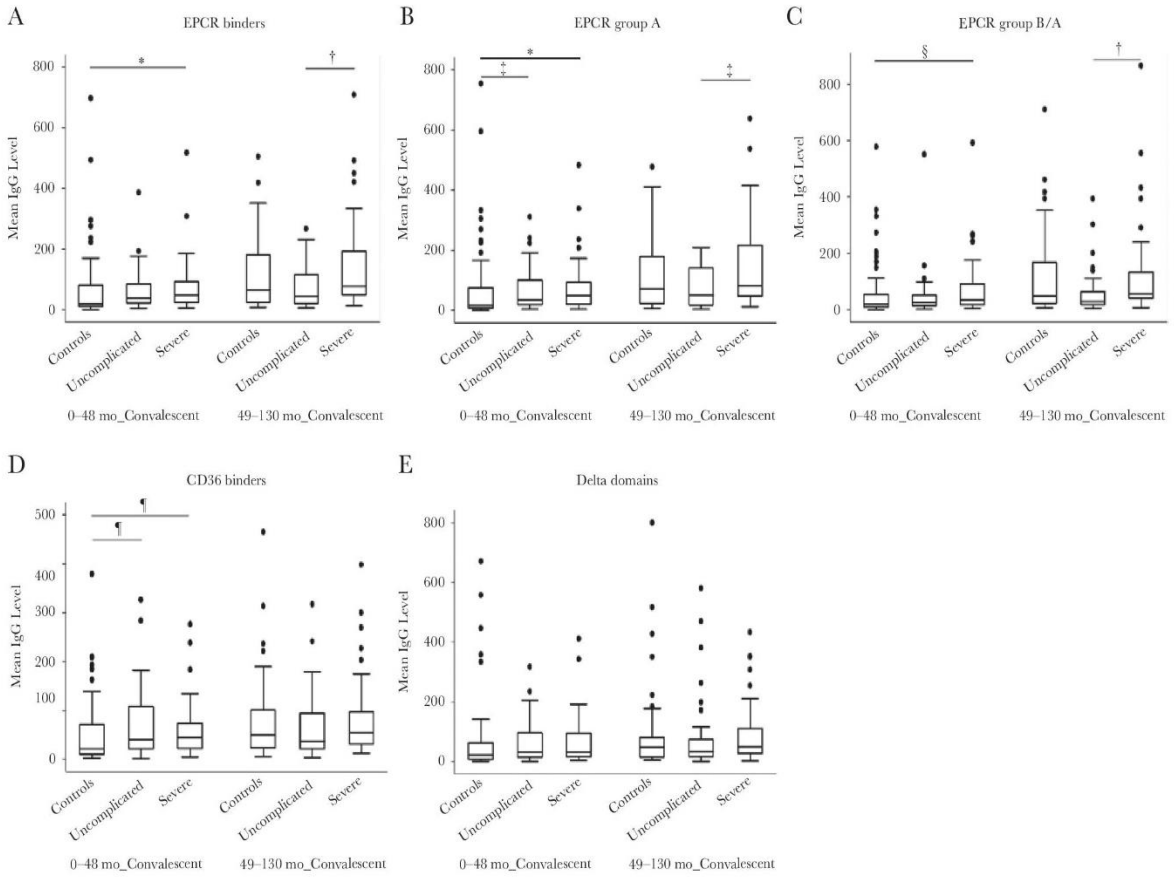

Figure 4. Levels of immunoglobulin G (IgG) against different cysteine-rich interdomain region (CIDR) domain types in convalescent-stage plasma samples from children in different disease severity groups and grouped according to age. Wilcoxon rank sum tests were used to compare community controls and participants with uncomplicated malaria or severe malaria (convalescent stage) in the 0–48-month age group; similar comparisons were done for participants in the 49–130-month age group. Values on the y-axes represent mean IgG levels in relative units; box plots show medians with interquartile range and outliers. Total number of participants in community control, uncomplicated convalescent malaria, and severe convalescent malaria groups: n = 64, 35, and 60, respectively, in the 0–48-month age group, and n = 47, 30, and 38, respectively, in the 49–130-month age group. Mean levels of IgG against the following CIDR domain types were measured: binding endothelial protein C receptor (EPCR) (*A*); binding EPCR, group A (*B*); binding EPCR, group B/A (*C*); binding cluster of differentiation 36 (CD36) (*D*); and delta domains associated with rosetting (*E*). $^{*}P = .01$; $\dagger P = .02$; $\ddagger P = .04$; $^{\S}P = .03$; $\P P = .048$.

controlling for exposure. Maturation of the immune system could also explain these age-stratified antibody responses [43].

Altogether, these data suggest that antibodies against EPCR-binding domains develop most prominently after severe malaria and that parasites causing severe malaria may be expressing EPCR-binding CIDRα1 domains. In a recent study from Mali, children with cerebral malaria or severe malarial anemia had limited repertoires of antibody against non–CD36-binding PfEMP1 at presentation, and convalescent serum samples showed more intense responses than acute serum samples to

Table 2. Changes Over Time in Levels of IgG Against Different CIDR Domain Types in the Severe Malaria Group (Convalescent vs Acute Stage) (n = 98)

	IgG Level, Median (IQR), Relative Units		
CIDR Domain Type	Severe Malaria, Acute Stage	Severe Malaria, Convalescent Stage	*P* Value[a]
CIDR EPCR	60.5 (25.3–102.8)	63.8 (29.1–118.4)	.04
CIDR EPCR group A	53.4 (25.5–106.0)	67.2 (26.9–116.9)	.02
CIDR EPCR group B/A	39.4 (18.5–94.9)	43.7 (21.2–93.6)	.13
CIDR CD36	44.9 (22.7–79.9)	49.5 (25.7–86.2)	.95
CIDR delta	41.1 (20.4–102.6)	44.1 (18.5–101.5)	.77

Abbreviations: CD36, cluster of differentiation 36; CIDR, cysteine-rich interdomain region; EPCR, endothelial protein C receptor; IgG, immunoglobulin G; IQR, interquartile range.

[a]The changes in IgG levels between the plasma samples obtained from participants with severe malaria at the baseline (acute) and follow-up (convalescent) periods were compared using the Wilcoxon matched-pairs signed rank test.

Table 3. Change Over Time in Levels of IgG Against Different CIDR Domain Types in the Severe Malaria Group Grouped by Age (Convalescent vs Acute Stage)[a]

CIDR Domain Type	Age Group, mo	IgG Level, Median (IQR), Relative Units		*P* Value[b]
		Severe Malaria, Acute Stage	Severe Malaria, Convalescent Stage	
CIDR EPCR	0–48	44.6 (19.6–89.3)	47.1 (21.7–93.2)	.53
	49–130	68.3 (28.7–121.5)	75.8 (47.3–199.3)	.02
CIDR EPCR group A	0–48	46.1 (17.6–95.5)	49.3 (19.5–94.2)	.39
	49–130	68.3 (29.2–123.6)	81.4 (45.9–223.3)	.01
CIDR EPCR group B/A	0–48	31.6 (13.8–81.3)	34.6 (15.6–91.9)	.81
	49–130	43.5 (24.5–140.2)	56.1 (38.2–145.6)	.04
CIDR CD36	0–48	38.7 (20.5–79.2)	44.7 (21.5–74.7)	.56
	49–130	53.0 (29.5–93.1)	54.5 (29.5–104.7)	.60
CIDR delta	0–48	35.1 (16.3–97.6)	32.4 (15.9–95.6)	.46
	49–130	50.5 (25.8–139.5)	50.8 (26.6–113.3)	.61

Abbreviations: CD36, cluster of differentiation 36; CIDR, cysteine-rich interdomain region; EPCR, endothelial protein C receptor; IgG, immunoglobulin G; IQR, interquartile range.

[a]There were a total of 60 participants with severe malaria in 0–48-month age group, and 38 in the 49–130-month age group.

[b]The changes in IgG levels between the plasma samples obtained from participants with severe malaria at the baseline (acute) and follow-up (convalescent) periods were compared using the Wilcoxon matched-pairs signed rank test.

non–CD36-binding PfEMP1 types and PfEMP1 types containing CIDRα1 domains. In that study, serum reactivity was measured against domains of CIDRα1-containing PfEMP1 types and not always the CIDRα1 domain itself [44]. Our results are consistent with findings of a study in Papuan adults, showing that protection from severe malaria was associated with exposure to a broad range of group A and B PfEMP1 types, whereas persons with uncomplicated malaria had antibodies against group C PfEMP1 [34]. The findings suggest that parasite phenotypes causing severe malaria in adults and children could be similar across different endemic settings.

In studies from African children [22, 23, 30] and Indian adults [31], parasites causing severe malaria express high levels of *var* genes encoding EPCR-binding PfEMP1, and antibodies against EPCR-binding CIDR domains are acquired earlier in life than antibodies to other CIDR domain types [29]. The link between PfEMP1 antibody reactivity and *var* gene expression was further demonstrated in a controlled human malaria infection study, in which parasite expression of group A and DC8-like PfEMP1 was associated with low levels of naturally acquired preexisting antibodies in participants [45].

In older children with uncomplicated malaria, levels of antibody against the EPCR-binding CIDRα1 domain types levels decreased in convalescent plasma. In children from Kilifi with uncomplicated malaria, antibody responses to surface antigens also declined during the 12 weeks after malaria episodes [46]. The presence of higher acute than convalescent levels of antibody to some PfEMP1 proteins in our study could be attributed to the relatively short-lived nature of some PfEMP1 antibody responses [46] and declining antibody levels could be due to lack of boosting by current infection. Future longitudinal studies will aid in resolving the dynamics of PfEMP1 antibody reactivity during infection and in convalescence.

In Tanzania, antibodies against CIDRγ/δ domains were acquired earlier than antibodies against CD36-binding CIDR domains [29]. The few PfEMP1 types mediating rosetting have all carried CIDRγ/δ domains, but the phenotype has not been directly linked to these domains [20]. We did not observe significant changes in antibody levels against CD36-binding domains or CIDRγ/δ domains in severe or uncomplicated malaria, suggesting that these variants were not prominently expressed in these infections. CIDRγ/δ type PfEMP1 may be less prevalent in PNG, because a putative rosetting receptor, complement receptor 1 [9], is commonly deficient in the PNG population [47]. Complement receptor 1–binding IE may not be able to sequester efficiently, possibly preventing a severe infection from being established and causing antibodies to be generated against them. Although some studies have shown high transcript levels of group A *var* genes in children with rosetting parasites in PNG [35], others have shown no associations between severe malaria and the rosetting rates [48].

Impaired consciousness, respiratory distress, and severe anemia are the commonly overlapping severe malaria syndromes in young African children. Of these, impaired consciousness and respiratory distress are the strongest predictors for mortality [49]. In these studies, respiratory distress (acidotic breathing) was considered a surrogate for metabolic acidosis, through increased respiratory drive. In the current study, we found that in convalescence, younger children with metabolic acidosis had higher levels of antibody against rosetting-associated CIDRδ domains than children without metabolic acidosis. In Kenyan children with severe malaria [50], the rosetting frequency of the infecting parasites was correlated with the degree of metabolic acidosis. This suggests that PfEMP1 types containing CIDRδ domains may be associated with metabolic acidosis, with rosette formation as a possible link [50]. In the same

study, it was shown that expression of group A–like *var* genes and the absence of rosetting were associated with impaired consciousness [50]. In the present study we also found that children with impaired consciousness had higher convalescent levels of antibody against group B/A CIDR EPCR domain types than children who had recovered from uncomplicated malaria, suggesting that these EPCR-binding CIDR domains may be associated with impaired consciousness in children with severe malaria.

Most studies of immunity to PfEMP1 were based in Africa. Strengths of our study include measurement of antibody to multiple PfEMP1 domains, providing novel insights into the types of PfEMP1 associated with severe malaria and individual severe malaria syndromes in PNG, development of antibodies against these types, and the influence of age on immunity in severe disease. The baculovirus and insect cell expression system used to produce the analyzed recombinant proteins reliably generate functional PfEMP1 domains with confirmations similar to the native PfEMP1. However, differences in glycosylation of the antigen may bias reactivity patterns [51]. Studies of antibody acquisition to defined antigens associated with malaria provide only an indirect indicator of parasite phenotypes causing disease. Possible study weaknesses include a lack of data on *var* gene expression, sample dropouts, and a restricted sample size that limited our ability to investigate how antibody responses differed between severe malaria syndromes. Further experiments are required to validate the functional roles of antibodies against the EPCR-binding domains and against IE expressing these domains, along with the specific IgG subclasses involved (reviewed in [24]). Studies investigating the blocking of the CIDRα1-EPCR binding by these antibodies would further corroborate our findings.

In summary, children with severe malaria had higher antibody levels in convalescence against the EPCR-binding group A or group B/A PfEMP1 domains than children with uncomplicated malaria, and these differences were largely restricted to older children. These data suggest that severe malaria in PNG is associated with parasites expressing EPCR-binding PfEMP1. Further studies are required to establish whether these antibodies confer protection against severe malaria.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

Notes

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Potential conflicts of interest. All authors: No reported conflicts. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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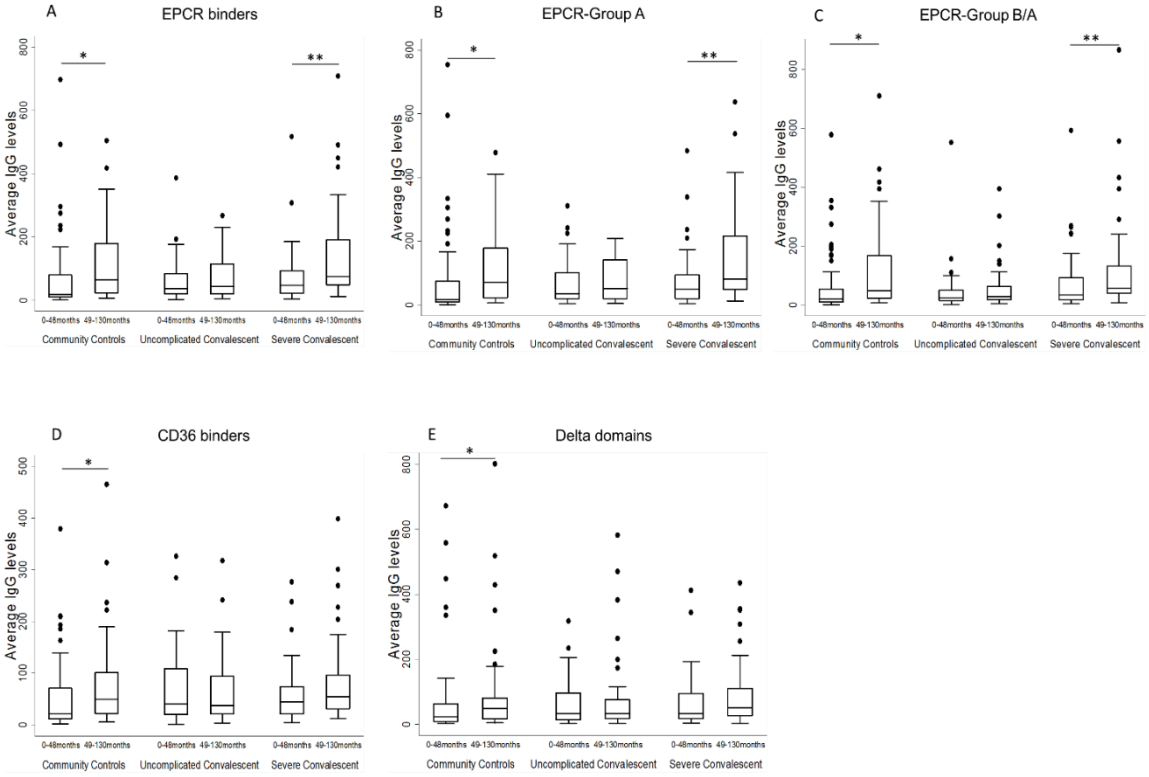
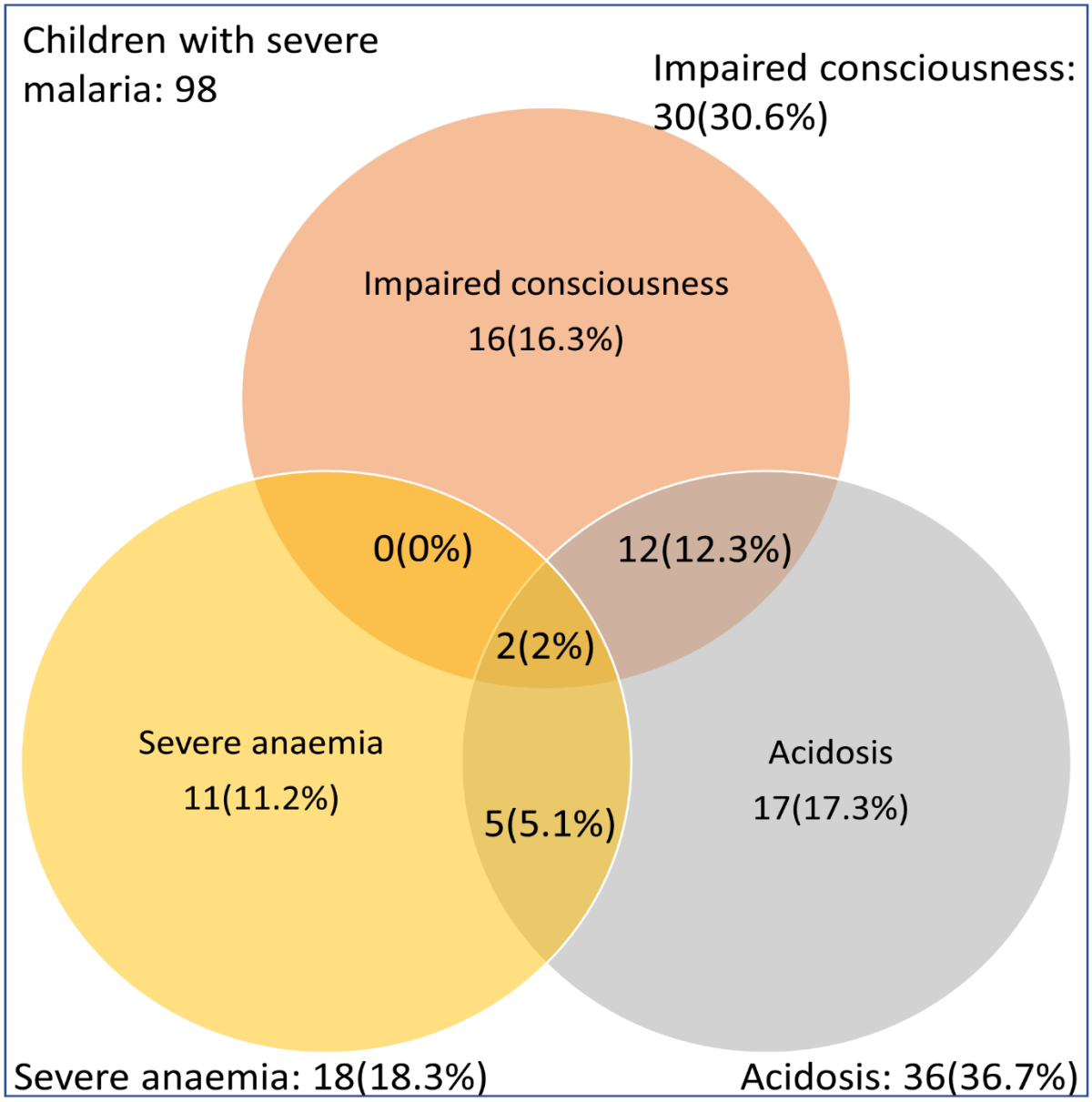
Supplementary data

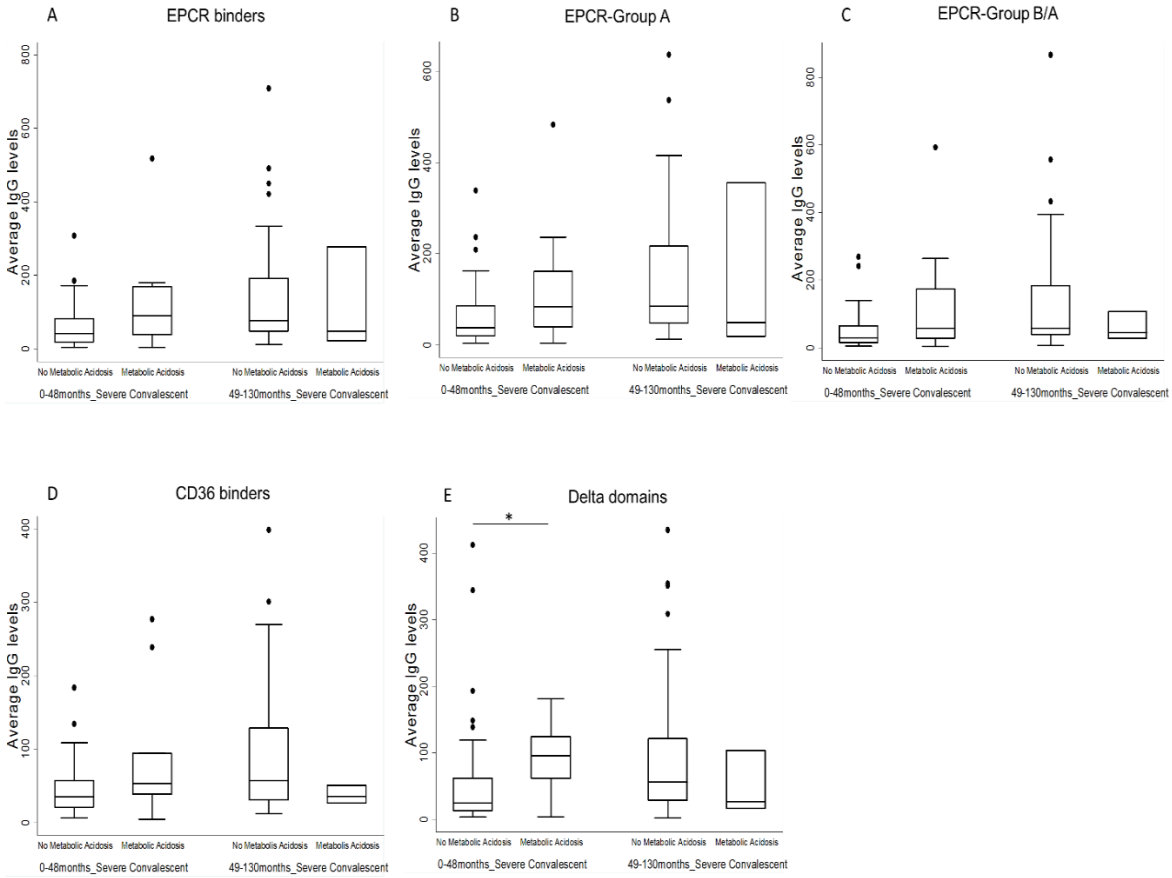
Supplementary figures



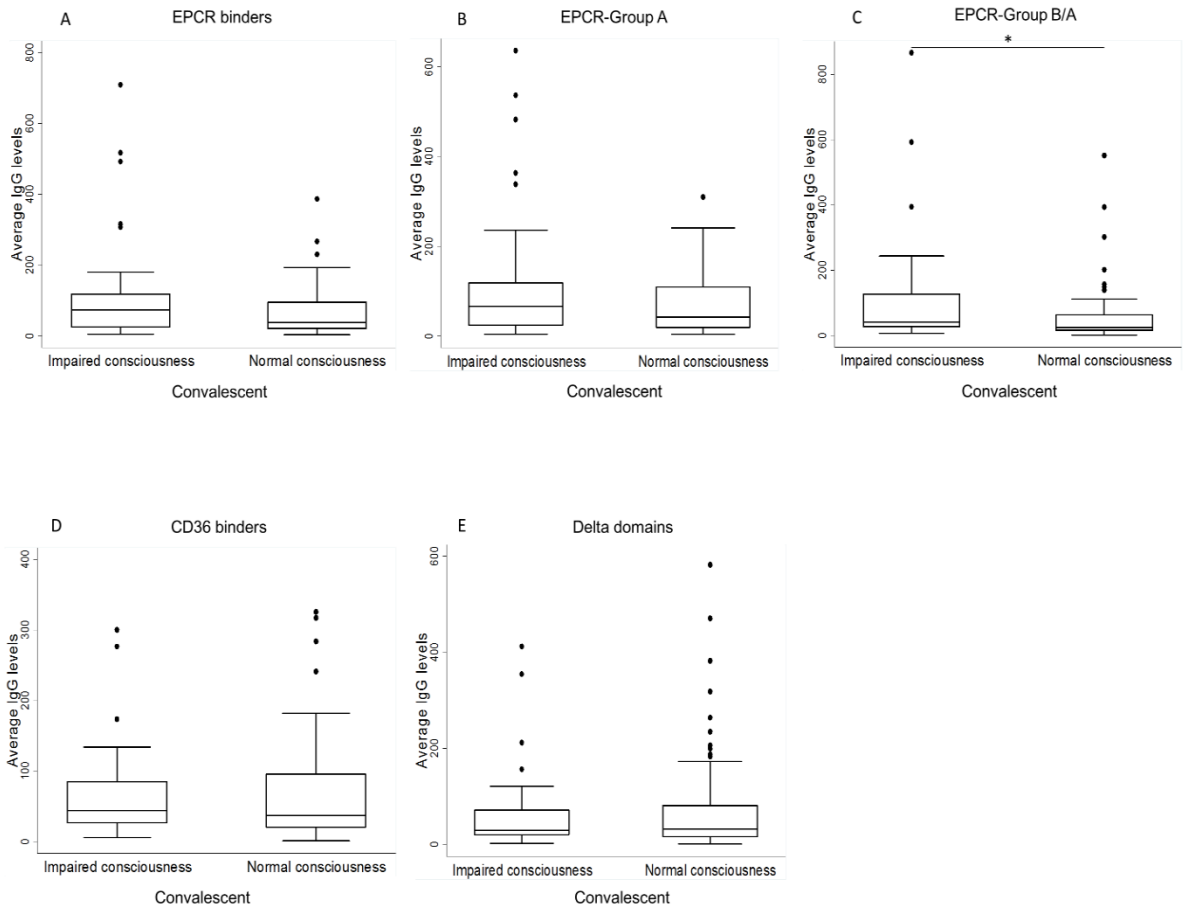
Supplementary Figure 1. IgG levels against different CIDR domain types, in convalescent plasma from children grouped according to age and belonging to different disease severity groups. Using Wilcoxon Rank Sum test, participants in the 0-48 months age group were compared to those in the 49-130 months age group for each disease severity group. Y-axes represent average IgG levels in Relative Units. Box plot shows median with lower 25th percentile and upper 75th percentile and outliers. Total number of participants in community controls group, uncomplicated convalescent malaria group and severe convalescent malaria group: n=64, 35, and 60 respectively in the 0-48 months age group; and n= 47, 30 and 38 respectively in the 49-130 months age group. Average IgG levels against the following CIDR domain types were measured: A: Binding EPCR, * p=0.0002, ** p=0.0.014. B: Binding EPCR and belonging to Group A, * p=0.0002 ** p=0.0129. C: Binding EPCR and belonging to Group B/A, * p=0.0004 ** p=0.0071. D: Binding CD36, * p=0.0094. E: Delta domains associated with rosetting, * p=0.0133. IgG, immunoglobulin G; CIDR, cysteine-rich inter-domain region; EPCR, endothelial protein C receptor; CD36, cluster of differentiation 36.



Supplementary Figure 2. Overlapping severe malaria syndromes in children with severe malaria. The overlapping severe malaria syndromes in children with severe malaria (n=98) have been described using a Venn diagram. Children with severe anaemia (n= 18), impaired consciousness (blantyre coma score, BCS 0-4) (n=30) and acidosis (n=36) and the number of children with overlapping syndromes are presented as total number (percentage). The acidosis circle includes children with respiratory distress and hyperlactataemia as these are often considered as surrogates for metabolic acidosis. Children with severe malaria not presenting with any of the specified severe malaria syndromes met other criteria set by WHO for severe malaria.



Supplementary Figure 3. IgG levels against different CIDR domain types in convalescent plasma from children with severe malaria and with or without metabolic acidosis, grouped according to age. Using Wilcoxon Rank Sum test, severe malaria participants within the 0-48 months age group were compared. Similar comparisons were made for the 49-130 months age group. Y-axes represent average IgG levels in Relative Units. Box plot shows median with lower 25th percentile and upper 75th percentile and outliers. Total number of participants, in the severe convalescent group without metabolic acidosis and with metabolic acidosis: n=46 and 13 respectively in the 0-48 months age group; and n= 35 and 3 respectively in the 49-130 months age group. Average IgG levels against the following CIDR domain types were measured: A: Binding EPCR. B: Binding EPCR and belonging to Group A. C: Binding EPCR and belonging to Group B/A. D: Binding CD36. E: Delta domains associated with rosetting, *p=0.0309. IgG, immunoglobulin G; CIDR, cysteine-rich inter-domain region; EPCR, endothelial protein C receptor; CD36, cluster of differentiation 36.



Supplementary Figure 4. IgG levels against different CIDR domain types in convalescent plasma from children with severe malaria with impaired consciousness and children with uncomplicated malaria with normal consciousness. Using Wilcoxon Rank Sum test, severe malaria participants with impaired consciousness (BCS score 0-4) and uncomplicated malaria participants with normal consciousness (BCS score 5) were compared. Y-axes represent average IgG levels in Relative Units. Box plot shows median with lower 25th percentile and upper 75th percentile and outliers. Total number of participants, in the severe convalescent group with impaired consciousness (BCS score 0-4), n= 30 and for uncomplicated convalescent group and normal consciousness (BCS score 5), n= 65. Average IgG levels against the following CIDR domain types were measured: A: Binding EPCR. B: Binding EPCR and belonging to Group A. C: Binding EPCR and belonging to Group B/A, * p=0.0211. D: Binding CD36. E: Delta domains associated with rosetting. IgG, immunoglobulin G; BCS, blantyre coma score; CIDR, cysteine-rich inter-domain region; EPCR, endothelial protein C receptor; CD36, cluster of differentiation 36.

Supplementary tables

Supplementary table 1: Summary of study population with severe malaria symptoms

Symptoms	Severe malaria (n=98)
Blantyre Coma Score:	
0	2.0 (2)
2	6.1 (6)
3	6.1 (6)
4	16.3 (16)
5	69.4 (68)
Severe anaemia	18.4 (18)
Respiratory distress	12.2 (12)
Blood lactate > 5 mmol/L	18.4 (18)
Metabolic acidosis	16.3 (16)

Data represented as percentages and (total number of children with each symptom).

Supplementary Table 2. Recombinant CIDR domains used for the Luminex assay.

Protein name[a]	Name used in figure	CIDR domain class	Domain Cassette	PfEMP1 group	Predicted binding phenotype	Genome/ Isolate	PfEMP1	CIDR domain types generated
CIDRα1.4_HB3var03_HB3 [1, 2]	α1.4 (a)	α1.4		A	EPCR	HB3	HB3var03	CIDR EPCR and Group A CIDR EPCR
CIDRα1.4_IT4var7_IT4 [1, 2]	α1.4 (b)	α1.4		A	EPCR	IT4	IT4var7	CIDR EPCR and Group A CIDR EPCR
CIDRα1.5a_1965_2_1965 [1, 3]	α1.5a (a)	α1.5a		A	EPCR	1965	1965_2	CIDR EPCR and Group A CIDR EPCR
CIDRα1.5a_GA013_ERS010323 [1, 4]	α1.5a (b)	α1.5a		A	EPCR	ERS010323	GA013	CIDR EPCR and Group A CIDR EPCR
CIDRα1.5a_GA014_ERS010022 [1, 4]	α1.5a (c)	α1.5a		A	EPCR	ERS010022	GA014	CIDR EPCR and Group A CIDR EPCR

CIDRα1.5b_1918_5_1918 [1, 5]	α1.5b (a)	α1.5b	A	EPCR	1918	1918_5	CIDR EPCR and Group A CIDR EPCR
CIDRα1.5b_1983_13_1983 [1, 3]	α1.5b (b)	α1.5b	A	unknown	1983	1983_13	CIDR EPCR and Group A CIDR EPCR
CIDRα1.6a_HB3var02_HB3 [1, 2]	α1.6a	α1.6a	A	EPCR	HB3	HB3var02	CIDR EPCR and Group A CIDR EPCR
CIDRα1.6b_GA018_ERS010570 [1, 4]	α1.6b (a)	α1.6b	A	EPCR	ERS010570	GA018	CIDR EPCR and Group A CIDR EPCR
CIDRα1.6b_GA019_ERS010031 [1, 4]	α1.6b (b)	α1.6b	A	EPCR	ERS010031	GA019	CIDR EPCR and Group A CIDR EPCR
CIDRα1.7_1965_8_1965 [1, 3]	α1.7 (a)	α1.7	A	EPCR	1965	1965_8	CIDR EPCR and Group A CIDR EPCR
CIDRα1.7_1918_3_1918	α1.7 (b)	α1.7	A	EPCR	1918	1918_3	CIDR EPCR and Group A

[1, 5]								CIDR EPCR
CIDRα1.7_GA024_ERS010438 **[1, 4]**	α1.7 (c)	α1.7		A	EPCR	ERS010438	GA024	CIDR EPCR and Group A CIDR EPCR
CIDRα1.1_IT4var20_IT4 **[1, 2]**	α1.1 (a)	α1.1	DC8	B/A	EPCR	IT4	IT4var20	CIDR EPCR and Group B/A CIDR EPCR
CIDRα1.1_igh_var19_IGH **[1, 2]**	α1.1(b)	α1.1	DC8	B/A	EPCR	IGH	igh_var19	CIDR EPCR and Group B/A CIDR EPCR
CIDRα1.1_raj116_var8_raj116 **[1, 2]**	α1.1 (c)	α1.1	DC8	B/A	EPCR	raj116	raj116_var8	CIDR EPCR and Group B/A CIDR EPCR
CIDRα1.8a_Ga026_ERS010178 **[1, 4]**	α1.8a	α1.8a	DC8	B/A	EPCR	ERS010178	Ga026	CIDR EPCR and Group B/A CIDR EPCR

CIDRα1.8b_Ga027_2053[b] [5]	α1.8b (a)	α1.8b	DC8	B/A	EPCR	2053	Ga027	CIDR EPCR and Group B/A CIDR EPCR
CIDRα1.8b_Ga029_ERS010532 [1, 4]	α1.8b (c)	α1.8b	DC8	BA	EPCR	ERS010532	Ga029	CIDR EPCR and Group B/A CIDR EPCR
CIDRα2.10_IT4var30_IT4 [2, 6]	α2.10	α2.10		B	CD36	IT4	IT4var30	CIDR CD36
CIDRα2.2_IT4var24_IT4 [2, 6]	α2.2	α2.2		B	CD36	IT4	IT4var24	CIDR CD36
CIDRα2.4_IT4var33_IT4 [2, 6]	α2.4	α2.4		B	CD36	IT4	IT4var33	CIDR CD36
CIDRα2.7_IT4var61_IT4 [2, 6]	α2.7	α2.7		B	CD36	IT4	IT4var61	CIDR CD36
CIDRα2.9_IT4var45_IT4	α2.9	α2.9		B	CD36	IT4	IT4var45	CIDR CD36

[2, 6]							
CIDRα3.1_DD2var01_DD2 [2, 6]	α3.1 (a)	α3.1	B	CD36	DD2	DD2var01	CIDR CD36
CIDRα3.1_HB3var27_HB3 [2, 7]	α3.1 (b)	α3.1	B	CD36	HB3	HB3var27	CIDR CD36
CIDRα3.1_IT4var21_IT4 [2, 7]	α3.1 (c)	α3.1	B	CD36	IT4	IT4var21	CIDR CD36
CIDRα3.3_IT4var26_IT4[c] [2]	α3.3	α3.3	B	CD36	IT4	IT4var26	CIDR CD36
CIDRα3.5_IT4var15_IT4 [2, 6]	α3.5	α3.5	B	CD36	IT4	IT4var15	CIDR CD36
CIDRα5_IT4var14_IT4 [2, 6]	α5	α5	B	CD36	IT4	IT4var14	CIDR CD36
CIDRα6_IT4var12_IT4 [2, 6]	α6	α6	B	CD36	IT4	IT4var12	CIDR CD36

CIDRδ_HB3var05_HB3 [2, 7]	δ (a)	δ	A	unknown	HB3	HB3var05	CIDR delta
CIDRδ_HB3var35_HB3 [2, 7]	δ (b)	δ	A	unknown	HB3	HB3var35	CIDR delta
CIDRδ_IT4var02_IT4 [2, 7]	δ (c)	δ	A	unknown	IT4	IT4var02	CIDR delta
CIDRγ_IT4var08_IT4 [2, 7]	γ	γ3	A	unknown	IT4	IT4var08	

CIDR, cysteine-rich inter-domain region; DC, domain cassette; EPCR, endothelial protein C receptor; CD36, cluster of differentiation 36.

[a] References for the protein production including amino acid sequence of expressed domains [1, 6, 7] and origin of sequence [2-5] included.

[b] Previously unpublished domain sequence: >CIDRα1.8_2053_3

PICGVKCENKSCTDKENDDDCKNKKKYDPPKGVTPIDIPILYSGDKQGDITKKLEDFCYNRTKENEKTYQNWKCYYKDSEFNKCKMESKSGKS
TTQEKIISFDEFFYLWVNNLLIDSIMWENDIKHCINNTNVTNCKNKCNENCKCFKNWVKKKEEEWTKVKQILGNRSENLNNYYNKLNSLFKGFF
FEVVYKFNNKEEKWNKLTEKLEQKIGSSKGKEGVENPKDAIELLLDHLKENAITCKDNNSLEEDKNCPKIKINPC

[C] Previously unpublished domain sequence: >CIDRα3.3_D3_IT4var26

PYCGMKKKGDNWVAKENDENCKRGNLYTILTNAESTNIDVLSFGDKREDRETKLIKFCAEKNGGVAGGGGSGSNSNSKELYEEWKCYKHDY
VKEVGEKDEDEEENLEKVKAAGGLCILKKEKKGVEETNSQKEPDEIQKTFNPFFYYWVAHMLKDSIYWETQKIKKCLKKGTKIKCTEKCKRDC
GCFKRWVEQKQTEWKAIKKHFKTQDNIIEGFHDITLKEVLKLEFENKNTEEDKENNVSAEEIDLINKMLKEDETAVAGASGGEDNTTIDKLLKHE
LDEAKQCIKKC

Supplementary Table 3. IgG levels against individual CIDR domains in participant groups at baseline (Severe acute Vs Uncomplicated acute[a]).

Individual CIDR domains	PfEMP1 group	Predicted binding phenotype	Z	P value[b]
CIDRα1.5b (b)	Group A	Unknown	2.081	0.0374
CIDRα1.8b (a)	Group B/A	EPCR	-2.179	0.0293
CIDRα3.3	Group B	CD36	-2.379	0.0173
CIDRγ3	Group A	Unknown	-2.005	0.0450

CIDR, cysteine-rich inter-domain region; EPCR, endothelial protein C receptor; CD36, cluster of differentiation 36.

[a] Total number of participants in each category: severe acute, n=98; uncomplicated acute, n=65.

[b] Using Wilcoxon Rank Sum test, participants with severe acute malaria were compared to those with uncomplicated acute malaria.

Supplementary Table 4: IgG levels against individual CIDR domains in participant groups at follow-up (Severe Convalescent Vs Uncomplicated Convalescent[a]).

Individual domains	CIDR	PfEMP1 group	Predicted binding phenotype	Z	P value[b]
CIDRα1.1 (b)		Group B/A	EPCR	2.350	0.0187
CIDRα1.1 (c)		Group B/A	EPCR	2.235	0.0254
CIDRα1.5a (b)		Group A	EPCR	2.228	0.0259
CIDRα1.5b (b)		Group A	unknown	2.342	0.0192
CIDRα1.7 (c)		Group A	EPCR	2.564	0.0103
CIDRα1.8 (a)		Group B/A	EPCR	2.418	0.0156
CIDRα1.8b (c)		Group B/A	EPCR	2.999	0.0027
CIDRα2.9		Group B	CD36	2.337	0.0194

CIDR, cysteine-rich inter-domain region; DC, domain cassette; EPCR, endothelial protein C receptor; CD36, cluster of differentiation 36.

[a] Total number of participants in each category: severe convalescent, n=98; uncomplicated convalescent, n=65

[b] Using Wilcoxon Rank Sum test, convalescent plasma from severe malaria group were compared to those with uncomplicated malaria.

Supplementary Table 5. Change in IgG levels over time in the uncomplicated malaria group against different CIDR domain types (Uncomplicated Convalescent Vs Uncomplicated acute[a]).

CIDR domain type	Uncomplicated malaria		
	Median [IQR]		P-value[b]
	Acute	Conv.	
CIDR EPCR	48.4 [22.3-117.3]	37.9 [19.9-98.2]	0.063
CIDR EPCR Group A	51.3 [21.4-126.4]	42.0 [19.1-111.1]	0.102
CIDR EPCR Group B/A	35.3 [20.5-83.3]	23.9 [15.2-62.7]	0.009
CIDR CD36	56.6 [25.9-104.1]	37.1 [20.5-100.4]	0.275
CIDR delta	44.9 [19.3-117.2]	32.3 [14.8-88.6]	0.107

CIDR, cysteine-rich inter-domain region; IQR, interquartile range; Conv, convalescent; EPCR, endothelial protein C receptor; CD36, cluster of differentiation 36.

[a] Total number of participants with uncomplicated malaria, n=65.

[b] Change in IgG levels between the plasma taken from participants with uncomplicated malaria at baseline (acute) and follow-up (convalescent) period was determined using Wilcoxon matched-pairs signed rank test.

Supplementary Table 6. Change in IgG levels over time in the uncomplicated malaria group against different CIDR domain types in participants grouped by age (Uncomplicated Convalescent Vs Uncomplicated Acute[a]).

CIDR domain type		Uncomplicated Malaria		
		Median [IQR]		P-value[b]
	Age group (months)	Acute	Conv.	
CIDR EPCR	0-48	46.1 [24.2-77.9]	37.6 [20.6-84.4]	0.594
	49-130	66.3 [20.3-148.3]	43.5 [19.7-118.1]	0.036
CIDR EPCR Group A	0-48	47.2 [20.4-96.7]	35.5 [19.2-100.2]	0.851
	49-130	73.9 [21.6-166.7]	51.0 [18.3-145.8]	0.026
CIDR EPCR Group B/A	0-48	35.3 [21.8-68.6]	23.5 [13.6-50.1]	0.030
	49-130	38.4 [18.1-110.2]	28.0 [17.6-68.6]	0.133
CIDR CD36	0-48	56.6 [26.7-106.2]	40.1 [20.3-108.5]	0.617
	49-130	55.6 [22.6-105.7]	36.9 [19.9-94.4]	0.355

CIDR delta	0-48	46.9 [22.0-99.9]	32.3 [13.2-95.7]	0.110
	49-130	41.1 [15.4-179.2]	33.2 [15.3—85.4]	0.565

CIDR, cysteine-rich inter-domain region; IQR, interquartile range; Conv, convalescent; EPCR, endothelial protein C receptor; CD36, cluster of differentiation 36.

[a] Total number of participants with uncomplicated malaria in 0-48 months age group: n=35 and in the 49-130 months age group: n=30

[b] Change in IgG levels between the plasma taken from participants with uncomplicated malaria at baseline (acute) and follow-up (convalescent) period was determined using Wilcoxon matched-pairs signed rank test.

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5 Chapter 5: Role of opsonic antibodies in immunity against certain PfEMP1 types in children with severe or uncomplicated malaria from Papua New Guinea

5.1 Introduction

PfEMP1s are the main targets of antibody immunity against malaria infection, while the other proteins expressed on the surface of the IE are minor targets [321]. Severe malaria is associated with a serologically conserved subset of VSA [213] and naturally acquired immunity against severe malaria is acquired after one to three malaria episodes [217]. The antibody repertoire against different PfEMP1 types however, only increases with repeated exposure to those types [218].

In chapter 4, we examined associations between acquisition of antibodies to certain PfEMP1 types -specifically, a set of the different CIDRα domains these proteins contain- and severe malaria in young children from PNG. The different CIDRα domains tested were those binding EPCR or CD36 or associated with rosetting. We provided evidence suggesting that the EPCR-binding CIDRα1 domains of PfEMP1 were associated with severe malaria, with antibody levels against these domains being higher in older children convalescent from severe malaria than uncomplicated malaria. Antibody levels against all PfEMP1 types were broadly similar at hospital presentation. Together, these results suggest that PfEMP1s containing the EPCR-binding CIDRα1 domains may be involved in the pathogenesis of severe malaria in PNG children.

Do functional antibodies against parasite IE expressing the entire native PfEMP1 differ by severity? Are functional antibodies associated with protection from severe malaria and severe malaria syndromes? These were some of the questions that we were interested in answering based on our results from chapter 4.

Naturally acquired antibodies against IE can have varied functions such as inhibiting adhesion of IE to host cell receptors, opsonic phagocytosis of the IE or inhibiting egress of parasites from schizonts (reviewed in [241]). The role of opsonising antibodies in immunity in children with severe malaria or against specific severe malaria syndromes is little studied.

Opsonic phagocytosis, that is, clearance of the IE opsonised by antibodies targeted mainly against PfEMP1s by monocytes and macrophages, is an important function of malarial antibodies. Comparatively, non-opsonic phagocytosis occurs to a lesser degree as was shown for IE binding the placenta [322]. The role of opsonising antibodies against

the VSAs implicated in placental malaria has been previously well studied, with studies showing their importance in parasite clearance and reduced risk of maternal anaemia [264] or loss of opsonic phagocytosis activity due to HIV co-infections and hence increase in the risk of pregnancy-associated malaria [265, 266]. In studies from adults with malaria, PfEMP1 was identified as a major target for the opsonising antibodies [190]. Recently, the same group also showed that in children with severe malaria from PNG (same as the study population used for our current aim), PfEMP1 was the major target of naturally acquired antibodies. These antibodies were associated with a reduced risk of severe malaria, functioned partly through opsonic phagocytosis, with PfEMP1 also being the dominant target of opsonising antibodies [323].

In this chapter, we have investigated differences in opsonising antibody levels between children with severe malaria and uncomplicated malaria at hospital presentation and during follow-up and in healthy age-matched community controls against IE binding different host endothelial receptors to identify responses that differed by disease severity. We also assessed if these antibodies are associated with protection from severe malaria. This in turn could help us infer the type of PfEMP1 expressed by parasites causing severe malaria in these areas and to determine whether our findings from chapter 4 also apply to measures of antibody against whole IE.

5.2 Methods

5.2.1 Study Population

Study participants were mainly from Madang region of Papua New Guinea. Children from Sepik and few other regions of PNG, living in Madang Province, were also included. Children aged 0.5-10 years who presented to the Modilon Hospital, Madang with either severe or uncomplicated malaria were included in the study. The study also included age-matched healthy individuals from the community (community controls) [324].

WHO based criteria were used for diagnosing children with severe malaria [325]. Participants were categorised as having severe malaria if they presented with one or more of the following symptoms: a Blantyre coma score (BCS) < 5 or impaired consciousness; severe anaemia (haemoglobin <50 g/L); respiratory distress; prostration; multiple seizures; hyperlactataemia (blood lactate > 5 mmol/L); hypoglycaemia (blood glucose ≤ 2.2 mmol/L); dark urine; jaundice; abnormal bleeding; persistent vomiting or signs of shock. Children presenting with malaria but having no criterion for severe malaria were categorised as having uncomplicated malaria. Participants in the community controls group were age- matched (± 12 months) to children presenting with malaria by

gender and ethnicity. In the controls, *P. falciparum* prevalence was 8.2%, while *P. vivax* prevalence was 14.1% by microscopy. In all children, *P. falciparum* infection was confirmed by light microscopy and speciation by nested PCR [324].

Venous blood was collected from each participant at two time points: presentation with severe or uncomplicated malaria (acute) and 8 weeks later (convalescent/ follow-up). Plasma was separated from the blood samples by centrifugation and stored at -70^{o}C until shipping to Melbourne. Thus, the samples were categorised into five groups: severe acute, severe convalescent, uncomplicated acute, uncomplicated convalescent and community controls.

A total of 1036 plasma samples were tested for opsonic antibody levels against the four parasite lines with different binding phenotypes (described in section 5.2.3.1). These samples included 233 children with severe acute malaria and 182 children from the severe convalescent group, 213 children with uncomplicated acute malaria and 172 children in the uncomplicated convalescent group and 236 community controls. Once the clinical database was available, several children from each group had to be excluded from the final analyses. Reasons for exclusion were presence of *P. vivax* infection (but not *P. falciparum*), absence of parasite densities recorded (either by PCR or microscopy) and absence of complete clinical information. Final analyses were carried out in 163 children with severe acute malaria, 158 children convalescent from severe malaria, 101 children with uncomplicated acute malaria, 99 children convalescent from uncomplicated malaria and 235 healthy individuals. Total number of samples tested, excluded and analysed have been summarised in table 5.1.

5.2.2 Ethics

Ethical approval was obtained by the PNG Institute of Medical Research Institutional Review board (IRB number 1103) and Medical Research Advisory Committee of the PNG Health Department (MRAC number 11.12). Informed written consent was provided by the children's parents or guardians and the study was carried out as per the principles of the Declaration of Helsinki [324].

Table 5.1 Summary of total number of samples from the study population tested, excluded and analysed for opsonic antibody levels.

Characteristic	Severe malaria		Uncomplicated malaria		Community controls
	Severe acute	Severe convalescent	Uncomplicated acute	Uncomplicated convalescent	
Total samples tested for opsonic phagocytosis	233[a]	182[b]	213[c]	172[d]	236[e]
Samples with *P. vivax* infections	15	15	37	36	
Samples with no parasite densities recorded	5	1	35	33	
Samples with missing clinical information	50	8	40	4	1
Total samples used for statistical analyses	163	158	101	99	235

Data represented as total number (n)

[a] for Var19 parasite line, number of samples tested: n=191 as we ran out of some samples, and excluded samples with *vivax* infections and *falciparum* densities=0 for the assay

[b] for Var19 parasite line, number of samples tested: n=157 as we ran out of some samples and excluded samples with *vivax* infections and *falciparum* densities=0 for the assay

[c] for Var19 parasite line, number of samples tested: n=150 as we ran out of some samples and excluded samples with *vivax* infections and *falciparum* densities=0 for the assay

[d] for Var19 parasite line, number of samples tested: n=112 as we ran out of some samples and excluded samples with *vivax* infections and *falciparum* densities=0 for the assay

[e] for Var19 parasite line, number of samples tested: n=211 as we ran out of some samples

5.2.3 Experimental methods

All laboratory methods used in this chapter have been descried in detail in chapter 3.

5.2.3.1 Parasite and cell cultures

Opsonic antibody levels were measured in children from PNG against four different 'parasite lines', each of which represents one dominant PfEMP1 binding phenotype. E8B3X parasite line [114] binds to CD36 and ICAM-1 receptor, P6A1 [136] is a CD36 binding parasite line, R29 is a rosetting parasite line [61] and Var19 parasite line [216] binds to EPCR. Erythrocytes infected with these parasites were cultured and maintained as described in section 3.2.1.

The IE were checked for their respective receptor binding phenotype as described in section 3.2.9. E8B3X was selected for ICAM-1 binding as described in section 3.2.10. The Var19 line was selected for EPCR binding using rabbit anti-Var19 antibody (generated by Prof. Joseph Smith, Center for Infectious Disease Research, Seattle, WA and provided by Prof. James Beeson, Burnet Institute, Melbourne) at the cell sorting facility at Melbourne Brain Centre, (The Florey Institute of Neuroscience and Mental Health) University of Melbourne, Parkville. Selection of Var19 IE for EPCR binding was carried out by Ms. Wina Hasang. The binding data for E8B3X and P6A1 as well as the Var19 phenotype data after sorting have been presented in appendix IV. R29 was selected for its phenotype using the rosette selection assay as described in section 3.2.7. Data for R29 is not presented, but ~50-60% rosettes was usually counted. Also, E8B3X is an ICAM-1/CD36 binder and based on previous qPCR data using E8B (selected for ICAM-1 binding) (done by Dr. Michael Duffy and group [326]) and the study by Lennartz and group [135] we think that this could be a group B ICAM-1 binder. For simplicity, the E8B3X IE line used in the present study has been referred as an ICAM-1 binder in this thesis. The exact PfEMP1/ *var* genes expressed by the parasite lines (used in the present study) at the time of experiment is not known and qPCR on these parasite lines is required to confirm the PfEMP1 types. The binding, rosette selection and post-sorting phenotype data were used as an indication of the possible PfEMP1 types.

The THP-1 cells are undifferentiated monocytic cells, which were cultured and maintained as described in section 3.3.1.

5.2.3.2 Measurement of total opsonic IgG levels using the phagocytosis assay

The IE were purified using the Percoll gradient method (section 3.2.8) and opsonic phagocytosis was carried out as described in section 3.8.2. The % phagocytosis was

measured in duplicates and duplicate samples showing more than 20% variance in their % phagocytosis levels were repeated.

5.2.4 Statistical Analyses

Antibodies measured using this assay are expressed as % phagocytosis, that is, proportion of DHE positive THP-1 cells that phagocytosed opsonised IE relative to those from the positive control. For simplicity, this % phagocytosis was used as an indirect measure of the opsonic antibody levels in the plasma samples tested, although actual antibody levels were not measured. Thus, the terms 'opsonic antibodies' or 'opsonising antibodies' have been used to describe and discuss the results. These antibody levels were also used as indirect measures of recognition of antigens similar to/ shared with the ones carried by parasites circulating in these regions. The data generated does not follow normal distribution, and hence different non-parametric tests were used for analyses. Corrections for the multiple comparisons carried out in this chapter were not made.

STATA13 (StataCorp LP, Texas, USA) and GraphPad Prism 5.0 (La Jolla, CA, USA) were used to carry out all the statistical analyses carried out in this chapter.

To determine the proportion of children with high opsonic antibody levels against different PfEMP1 types tested, we divided the population into two groups (for each PfEMP1 type tested) based on the median % phagocytosis levels: individuals with % phagocytosis levels > the median were High responders, while those having % phagocytosis levels ≤ the median were Low responders. The median % phagocytosis level for each parasite line was calculated by combining the % phagocytosis levels from the five severity groups: severe acute, severe convalescent, uncomplicated acute, uncomplicated convalescent and the community controls. This median level was different for the four different parasite lines tested. The proportion of children in these two responder groups were compared between children with different disease severity groups using a 2 X 2 chi^2 test. The null hypothesis was that there are no differences between the % High responders in the severe malaria group and the uncomplicated malaria group.

While the above chi^2 test was a qualitative type of analysis to get a broad idea of the differences in antibody levels against different PfEMP1 types, we next compared levels of total opsonic antibodies (% opsonic phagocytosis) between children with severe malaria and uncomplicated malaria for each of the PfEMP1 types, first at acute time point and then during convalescence. A Wilcoxon Rank Sum test was used for these analyses. Antibody levels were also compared between severe acute and convalescent groups and between uncomplicated acute and convalescent groups. A Wilcoxon matched-pairs

signed rank test was used in this case as the samples are paired (plasma from the same child was collected at the two time points).

A Wilcoxon Rank Sum test was used to compare the opsonic antibody levels between children with severe malaria (and a specific severe malaria syndrome) and children with uncomplicated malaria. The Wilcoxon Rank Sum test was also used to compare antibody responses between two severe malaria syndromes, while a Kruskal Wallis test was used to compare antibody levels across all three syndromes. For all the severe syndromes analyses, it is important to note that a child having overlapping/ multiple severe syndromes would be present in each of the individual syndrome analysis as well. For example, the same child with severe anaemia and impaired consciousness would be included in the severe anaemia group as well as the impaired consciousness group in the analyses.

5.3 Results

5.3.1 Summary of study population

At hospital presentation, plasma from 163 children with severe acute malaria and 158 children convalescent from severe malaria were analysed for differences in antibody levels against the four PfEMP1 types. Similar analyses were done on 101 acute plasma samples and 99 convalescent plasma samples from children with uncomplicated malaria, while plasma from 235 healthy children were used as community controls. The same children who presented with severe or uncomplicated malaria were also followed up for collection of plasma 8 weeks later. However, there are fewer children in the convalescent groups due to some children dropping out of the program or being unavailable (Table 5.2).

Children with severe malaria had a median age of 43 months with 44% being females and 55% being males. Children with uncomplicated malaria had a median age of 47 months, 42% were females and 57% males. Children from the community controls group were matched by age and ethnicity to children with malaria. They had a median age of 41 months (Table 5.2). Some of the severe malaria syndromes that affected children whose samples were tested in this chapter are summarised in table 5.3, while the overlapping severe malaria syndromes have been represented as a Venn diagram in figure 5.1.

Table 5.2 Summary of study population categorised by disease severity.

Characteristic	Severe malaria		Uncomplicated malaria		Community controls
	Severe acute (n=163)	Severe convalescent (n=158)	Uncomplicated acute (n=101)	Uncomplicated convalescent (n=99)	(n=235)
Age (months)[a]	43 [30-58]	42.5 [30-58]	47 [31-59]	47 [31-60]	41 [28-58]
Sex[b]					
(Female)	44.8	44.3	42.6	42.4	
(Male)	55.2	55.7	57.4	57.6	
Ethnicity[b]					
(Madang)	74.9	74.7	88.1	87.9	80.0
(Madang Sepik)[c]	9.8	10.1	5.0	5.0	12.8
(Other)	5.5	5.1	5.9	6.1	
(Sepik)	9.8	10.1	1.0	1.0	7.2
Haemoglobin (g/L)[a]	79 [54-92]		89 [72.5-100][d]		105 [95-114][e]
Initial *P. falciparum* density (parasites/µl)[a]	83427 [13935-181072]		23070 [4888-66053]		N/A

[a] Data provided for age, haemoglobin and initial *P. falciparum* density are the median values and [interquartile range]. Data as recorded at acute timepoint

[b] Sex and ethnicity are represented by percentages. Data as recorded at acute timepoint. Sex and ethnicity of the children from community controls group were matched to the children presenting with malaria.

[c] Children with Madang Sepik ethnicity represent children with mixed ethnicities.

[d] Uncomplicated acute, n=100 for haemoglobin levels

[e] Community controls, n=233 for haemoglobin levels

Table 5.3 Summary of severe malaria syndromes in children with severe malaria

Symptoms	Severe malaria
	Acute (n=163)
Blantyre Coma Score:	
0	1.8 (3)
1	1.2 (2)
2	8.6 (14)
3	6.1 (10)
4	17.2 (28)
5	65.0 (106)
Severe anaemia	20.9 (34)
Respiratory distress	11.7 (19)
Blood lactate > 5 mmol/L	19.6 (32) [a]
Metabolic acidosis	11.7 (19) [b]

Data represented as percentages and (total number of children with each symptom) as collected during acute time point. Number of children with overlapping severe malaria syndromes presented in figure 5.1.

[a] Severe acute, n= 162 for Blood lactate > 5 mmol/L

[b] Severe acute, n= 162 for Metabolic acidosis

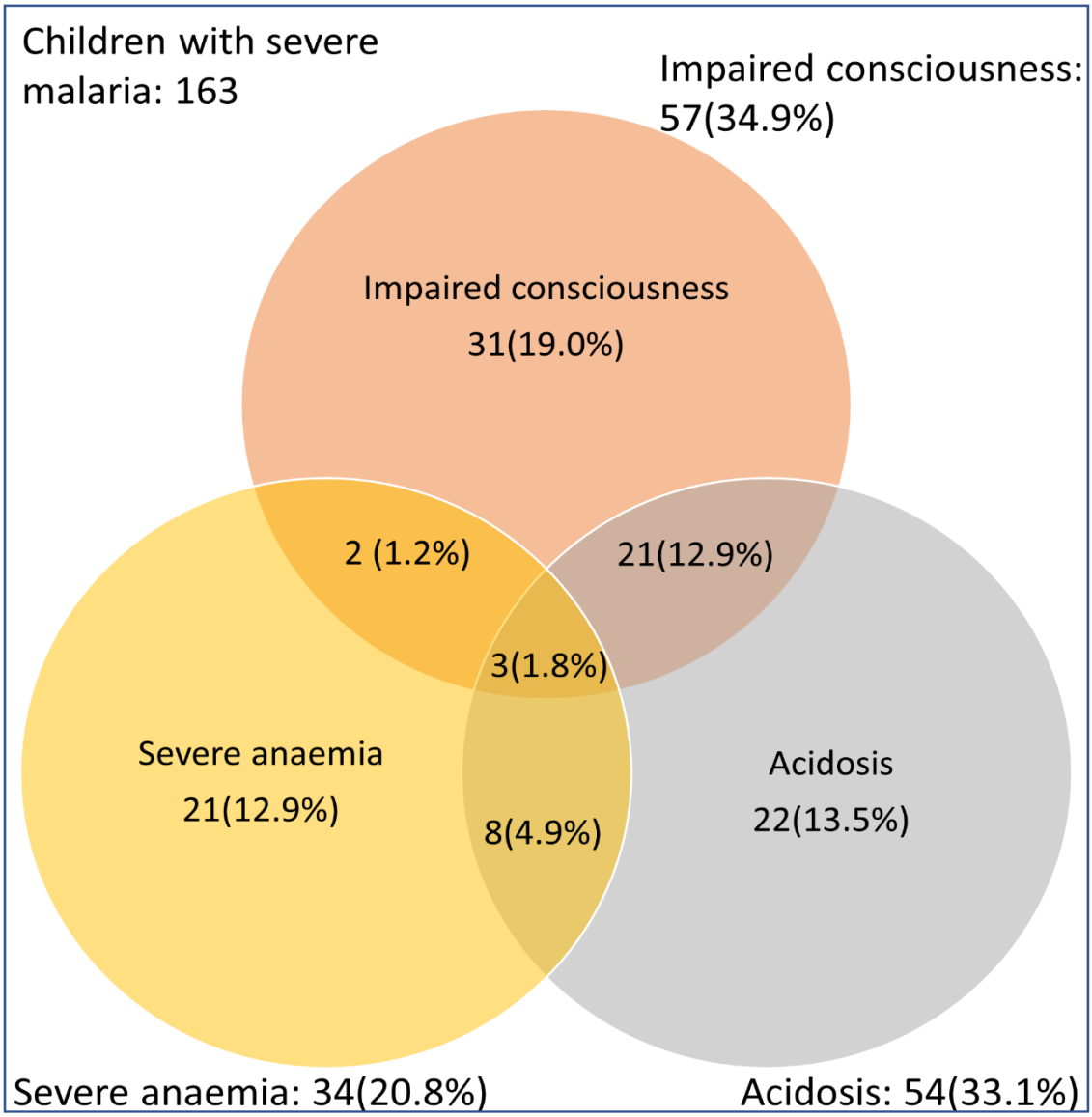


Figure 5.1 Overlapping severe malaria syndromes in children with severe malaria.
The most important overlapping severe malaria syndromes in children with severe malaria at presentation (n=163) have been described using a Venn diagram. Children with severe anaemia (n= 34), impaired consciousness (Blantyre coma score, BCS 0-4) (n=57) and acidosis (n=54) and the number of children with overlapping syndromes are presented as total number (percentage). The acidosis circle includes children with respiratory distress and hyperlactataemia as these are often considered as surrogates for metabolic acidosis. Children with severe malaria not presenting with any of the specified severe malaria syndromes met other criteria set by WHO for severe malaria [324, 325].

5.3.2 Proportion of children with high opsonic antibody responses against different PfEMP1 types at presentation and during convalescence

To determine the proportion of children with high opsonic antibody levels against different PfEMP1 types tested, we divided the population into two groups (for each PfEMP1 type tested) based on the median % phagocytosis levels: individuals with % phagocytosis levels > the median were High responders, while those having % phagocytosis ≤ the median were Low responders. We determined if the proportion of malaria participants with high opsonic antibody responses against the four PfEMP1 types: ICAM-1 binders, CD36 binders, PfEMP1 associated with rosetting and EPCR binders varied significantly across the different disease severity groups. Proportions of high opsonic antibody responders were compared for children with severe acute malaria and uncomplicated acute malaria, severe and uncomplicated malaria at convalescence and children with either category of malaria and age-matched community controls for the four PfEMP1 types.

5.3.2.1 ICAM-1-binding PfEMP1-E8B3X parasite line

Proportions of children with severe or uncomplicated malaria who were high antibody responders at presentation

We first divided antibody responses to E8B3X into High and Low responders, as described above. At presentation, the proportions of children with severe or uncomplicated malaria who were high responders was not significantly different (Chi2 = 2.90, p = 0.089). Community controls had significantly lower % High responders than children with severe or uncomplicated malaria at presentation (Chi^2 = 115.28 and 63.09 respectively and p < 0.001 for both) (Figure 5.2A).

Proportions of children with severe or uncomplicated malaria who were high antibody responders at convalescence

In convalescence, the % High responders against E8B3X did not differ significantly between children who had severe or uncomplicated malaria (Chi^2 = 1.49, p = 0.221). Community controls had significantly lower % High responders than children convalescent from severe or uncomplicated malaria (Chi^2 = 94.06 and 96.64 respectively and p < 0.001 for both) (Figure 5.2A).

5.3.2.2 CD36-binding PfEMP1-P6A1 parasite line

Proportions of children with severe or uncomplicated malaria who were high antibody responders at presentation

We first divided antibody responses to P6A1 into High and Low responders, as described above. At presentation, the proportion of children with severe malaria who were high responders was significantly higher than the proportion of children with uncomplicated malaria who were high antibody responders ($Chi^2 = 7.71$, $p = 0.006$). Community controls had significantly lower % High responders than children with severe or uncomplicated malaria at presentation ($Chi^2 = 55.93$ and 13.52 respectively and $p < 0.001$ for both) (Figure 5.2B).

Proportions of children with severe or uncomplicated malaria who were high antibody responders at convalescence

In convalescence, the % High responders against P6A1 did not differ significantly between children who had severe or uncomplicated malaria ($Chi^2 = 0.14$, $p = 0.708$). Community controls had significantly lower % High responders than children convalescent from severe or uncomplicated malaria ($Chi^2 = 5.63$, $p = 0.018$ and $Chi^2 = 2.70$, $p < 0.001$ respectively) (Figure 5.2B).

5.3.2.3 PfEMP1 associated with rosetting- R29 parasite line

Proportions of children with severe or uncomplicated malaria who were high antibody responders at presentation

We first divided antibody responses to R29 into High and Low responders, as described above. At presentation, the proportion of children with severe or uncomplicated malaria who were high responders was not significantly different ($Chi^2 = 0.32$, $p = 0.574$). Community controls had significantly lower % High responders than children with severe or uncomplicated malaria at presentation ($Chi^2 = 25.84$ and 24.71 respectively and $p < 0.001$ for both) (Figure 5.2C).

Proportions of children with severe or uncomplicated malaria who were high antibody responders at convalescence

In convalescence, the % High responders against R29 did not differ significantly between children who had severe or uncomplicated malaria ($Chi^2 = 3.31$, $p = 0.069$). Community controls had significantly lower % High responders than children convalescent from

severe or uncomplicated malaria (Chi^2 = 11.13 and 23.16 respectively and $p < 0.001$ for both) (Figure 5.2C).

5.3.2.4 EPCR-binding PfEMP1- Var19 parasite line

Proportions of children with severe or uncomplicated malaria who were high antibody responders at presentation

We first divided antibody responses to Var19 into High and Low responders, as described above. At presentation, the proportion of children with uncomplicated malaria who were high responders was significantly higher than the proportion of children with severe malaria who were high antibody responders (Chi^2 = 28.30, $p < 0.001$). Community controls had significantly lower % High responders than children with severe or uncomplicated malaria at presentation (Chi^2 = 17.58 and 85.24 respectively and $p < 0.001$ for both) (Figure 5.2D).

Proportions of children with severe or uncomplicated malaria who were high antibody responders at convalescence

In convalescence, the proportion of children with uncomplicated malaria who were high responders was significantly higher than the proportion of children with severe malaria who were high antibody responders (Chi^2 = 9.94, $p = 0.002$). Community controls had significantly lower % High responders than children with severe or uncomplicated malaria at convalescence (Chi^2 = 83.17 and 112.17 respectively and $p < 0.001$ for both) (Figure 5.2D).

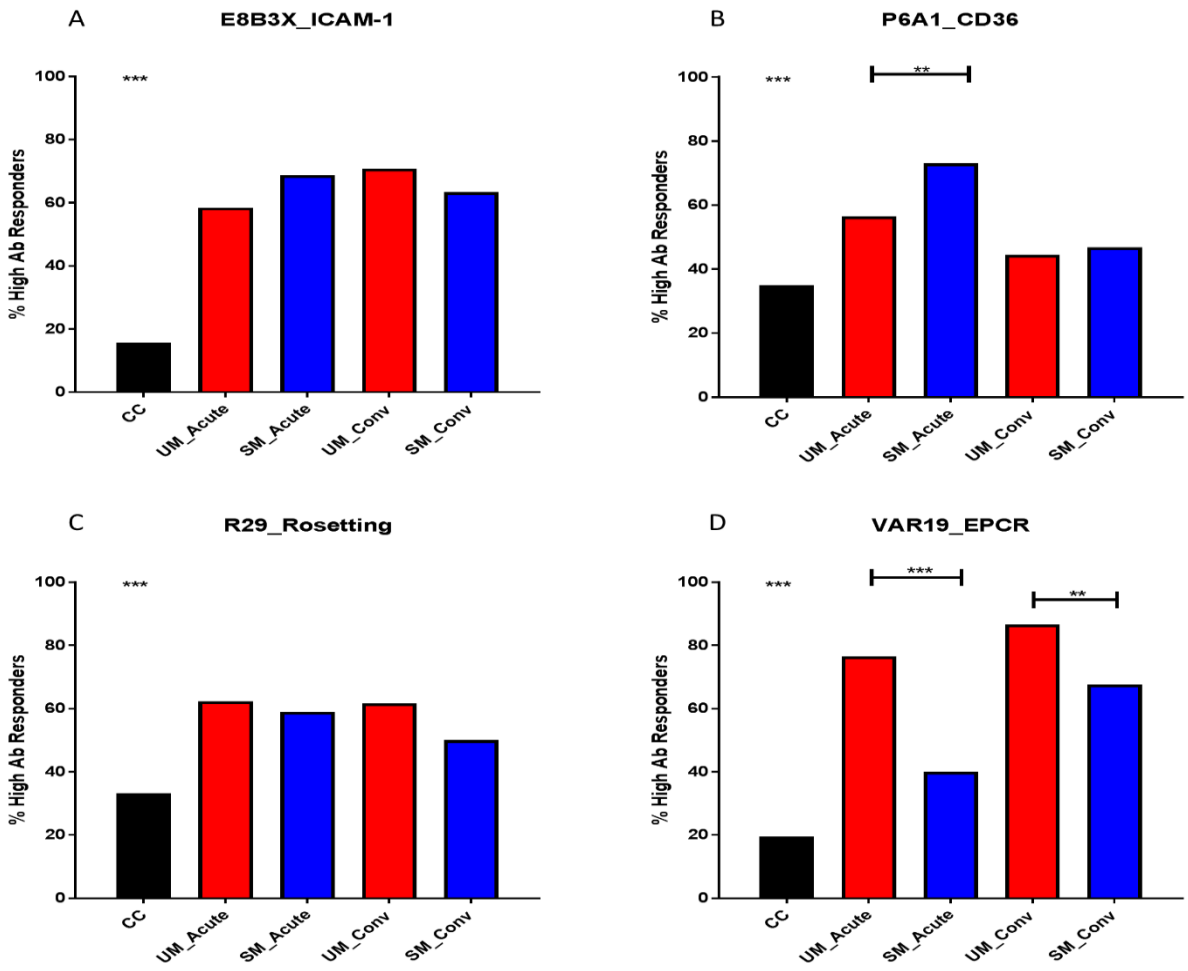


Figure 5.2 Proportion of children who were high opsonic antibody responders against different PfEMP1 types.

The population was divided into two groups (for each PfEMP1 type tested) based on the median % phagocytosis levels: High responders and Low responders. Individuals with % phagocytosis levels > the median were High responders, while those having % phagocytosis levels ≤ the median were Low responders. Using Chi^2 test, percent high antibody responders were compared between children with severe malaria and uncomplicated malaria at acute and convalescent time points and community controls. Data presented as bar graphs and Y-axis represents the percent high antibody responders. Significant associations indicated by p-values: * < 0.05, ** < 0.01, *** <0.001, **** < 0.0001. Total number of participants in the severe acute group, n = 163; severe convalescent group, n = 158; uncomplicated acute malaria group, n= 101 and uncomplicated convalescent group, n = 99. Percent high antibody responders in the different disease severity groups were compared against four PfEMP1 types: A. E8B3X, binding ICAM-1. B. P6A1, binding CD36. C. R29, associated with rosetting. D. VAR19, binding EPCR. Ab, antibody; CC, community controls; UM, uncomplicated malaria; SM, severe malaria; Conv, convalescent.

5.3.3 Opsonic antibody levels against different PfEMP1 types at presentation and during convalescence

We compared opsonising antibody levels in children with severe and uncomplicated malaria. For simplicity, the % phagocytosis measured using this assay was used as an indirect measure of the opsonic antibody levels in the plasma samples tested, although actual antibody levels were not measured. These antibody levels were also used as indirect measures of recognition of antigens similar to/ shared with the ones carried by parasites circulating in these regions. Differences in the levels of opsonic antibodies between the different disease severity groups in acute and convalescent plasma were used to investigate whether these antibodies might have a functional role in protection from severe malaria. Antibody levels were measured against PfEMP1 binding ICAM-1, CD36, associated with rosetting or binding EPCR.

5.3.3.1 ICAM-1-binding PfEMP1-E8B3X parasite line

Opsonic antibody levels at presentation in severe and uncomplicated malaria:

We first determined if the opsonic antibody levels differed between children with severe malaria and uncomplicated malaria at hospital presentation for the E8B3X line. Children with severe malaria had higher antibody levels as compared to those with uncomplicated malaria ($z = 2.2$; $p = 0.028$). The community controls had significantly lower levels of opsonic antibodies as compared to children with either severe or uncomplicated malaria ($z = -12.4$, $z = -10.7$ respectively; $p < 0.0001$ for both) (Figure 5.3).

Opsonic antibody levels at convalescence in severe and uncomplicated malaria:

On comparing the levels of opsonic antibody against E8B3X between children with severe malaria and uncomplicated malaria at convalescence, the antibody levels were found to be similar ($z = -0.8$; $p = 0.432$). As with acute time point, the community controls had lower opsonic antibody levels than the children with severe or uncomplicated malaria during convalescence ($z = -12.5$, $z = -11.6$ respectively; $p < 0.0001$ for both) (Figure 5.3).

Change in opsonic antibody levels from presentation to convalescence in severe and uncomplicated malaria

Opsonic antibody levels against E8B3X were compared in plasma from children with severe malaria at presentation and during convalescence. Similar comparisons were made for children with uncomplicated malaria. While in children with severe malaria, antibody levels did not change significantly over time ($p = 0.181$), children with

uncomplicated malaria showed an increase in antibody levels from acute to convalescence (p = 0.018) (Figure 5.3).

5.3.3.2 CD36-binding PfEMP1-P6A1 parasite line

Opsonic antibody levels at presentation in severe and uncomplicated malaria:

When opsonic antibody levels were compared between children with acute severe malaria and uncomplicated malaria, no significant differences in the antibody levels to P6A1 were observed (z = 0.8; p = 0.438). The community controls had lower antibody levels as compared to children with severe or uncomplicated malaria at presentation (z = -7.4, z = -4.2 respectively; p < 0.0001) (Figure 5.4).

Opsonic antibody levels at convalescence in severe and uncomplicated malaria:

Similar to results from acute plasma, even during convalescence, opsonic antibody levels did not differ between children with severe or uncomplicated malaria for P6A1 (z = 1.9; p = 0.063). The community controls children had lower opsonic antibody levels than children with severe malaria (z = -4.2; p < 0.0001), but no significant differences were observed when compared to children with uncomplicated malaria (z = -1.3; p = 0.201) (Figure 5.4).

Change in opsonic antibody levels from presentation to convalescence in severe and uncomplicated malaria

When we compared change in opsonic antibody levels from presentation to convalescence in the two disease severity groups, we found that the antibody levels against P6A1 declined over time for both groups: children with severe malaria and uncomplicated malaria (p < 0.0001 and p = 0.003 respectively) (Figure 5.4).

5.3.3.3 PfEMP1 associated with rosetting- R29 parasite line

Opsonic antibody levels at presentation in severe and uncomplicated malaria:

At hospital presentation, opsonic antibody levels to R29 were similar in children with severe and uncomplicated malaria (z = -1.1; p = 0.266). Children from the community controls group had lower antibody levels when compared to children with either severity of malaria (z = -6.3; p < 0.0001 for severe malaria, z = -5.6; p < 0.0001 for uncomplicated malaria) (Figure 5.5).

Opsonic antibody levels at convalescence in severe and uncomplicated malaria:

At convalescence, children with uncomplicated malaria had higher opsonic antibody levels than children with severe malaria against R29 line ($z = 2.4$; $p = 0.015$). The community controls had significantly lower levels of opsonic antibodies as compared to children with severe ($z = -4.9$; $p < 0.0001$) and uncomplicated malaria ($z = -5.8$; $p < 0.0001$) (Figure 5.5).

Change in opsonic antibody levels from presentation to convalescence in severe and uncomplicated malaria

When opsonising antibody levels at presentation were compared with antibody levels at convalescence for R29, the levels remained similar for children with uncomplicated malaria ($p = 0.357$). However, for children with severe malaria, the antibody levels dropped over time ($p = 0.013$) (Figure 5.5).

5.3.3.4 EPCR-binding PfEMP1- Var19 parasite line

Opsonic antibody levels at presentation in severe and uncomplicated malaria:

At presentation, opsonic antibody levels for Var 19 were significantly higher in children with uncomplicated malaria when compared to those with severe malaria ($z = 6.0$; $p < 0.0001$). On comparing the opsonic antibody levels in children with acute malaria and the community controls, we found the children from the controls group had lower antibody levels than children with either type of malaria ($z = -2.5$; $p = 0.012$ for severe malaria and $z = -9.5$; $p < 0.0001$ for uncomplicated malaria) (Figure 5.6).

Opsonic antibody levels at convalescence in severe and uncomplicated malaria:

In the plasma collected at convalescence, the opsonic antibody levels against Var19 were higher in children with uncomplicated malaria as compared to children with severe malaria ($z = 4.3$; $p < 0.0001$), which is similar to the results obtained at acute time point. The community controls had lower opsonic antibody levels then children with either severe or uncomplicated malaria ($z = -10.5$, $z = -11.3$ respectively; $p < 0.0001$ for both) (Figure 5.6).

Change in opsonic antibody levels from presentation to convalescence in severe and uncomplicated malaria

When opsonic antibody levels were compared over time, children with severe malaria showed an increase in antibody levels from presentation to convalescence for Var19 ($p = 0.0007$). However, in case of uncomplicated malaria, the antibody levels remained similar with time ($p = 0.478$) (Figure 5.6).

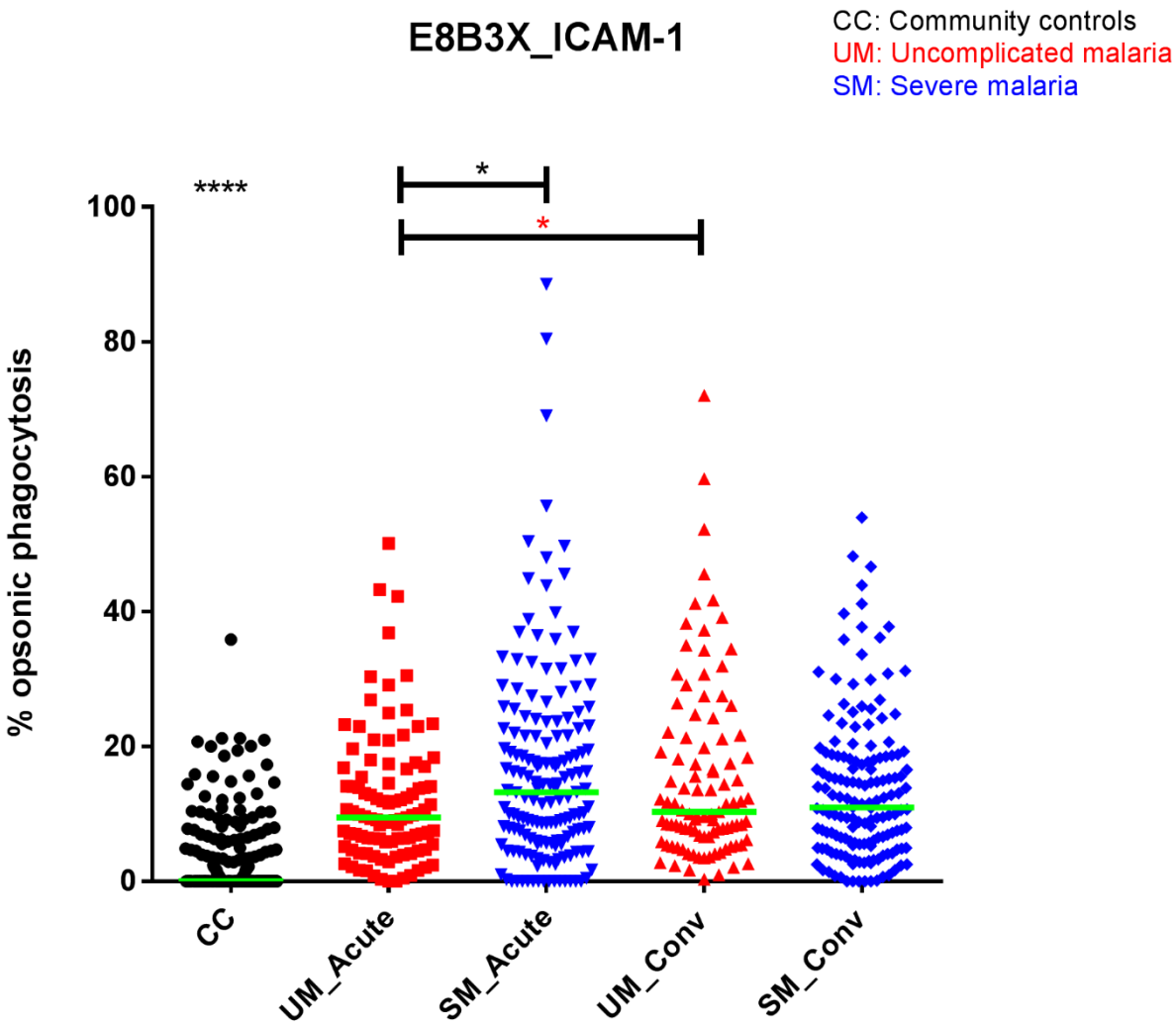


Figure 5.3 Opsonic antibody levels against ICAM-1-binding PfEMP1 (E8B3X parasite line) in acute and convalescent plasma.

Using a Wilcoxon Rank Sum (for unpaired samples) or Wilcoxon matched-pairs signed rank test (for paired samples), antibody levels were compared between children with severe malaria and uncomplicated malaria at acute and convalescent time points and community controls. Y-axis represents the percent opsonic phagocytosis, that is, percentage of THP-1 cells that have phagocytosed the infected erythrocyte relative to the positive control. Median antibody levels are presented as green horizontal bars for each disease severity group. Significant associations indicated by p-values: * < 0.05, ** < 0.01, *** <0.001, **** < 0.0001 (black *: unpaired test, red *: paired test). Total number of participants in the severe acute group, n = 163; severe convalescent group, n = 158; uncomplicated acute malaria group, n= 101 and uncomplicated convalescent group, n = 99. CC, community controls; UM, uncomplicated malaria; SM, severe malaria; Conv, convalescent.

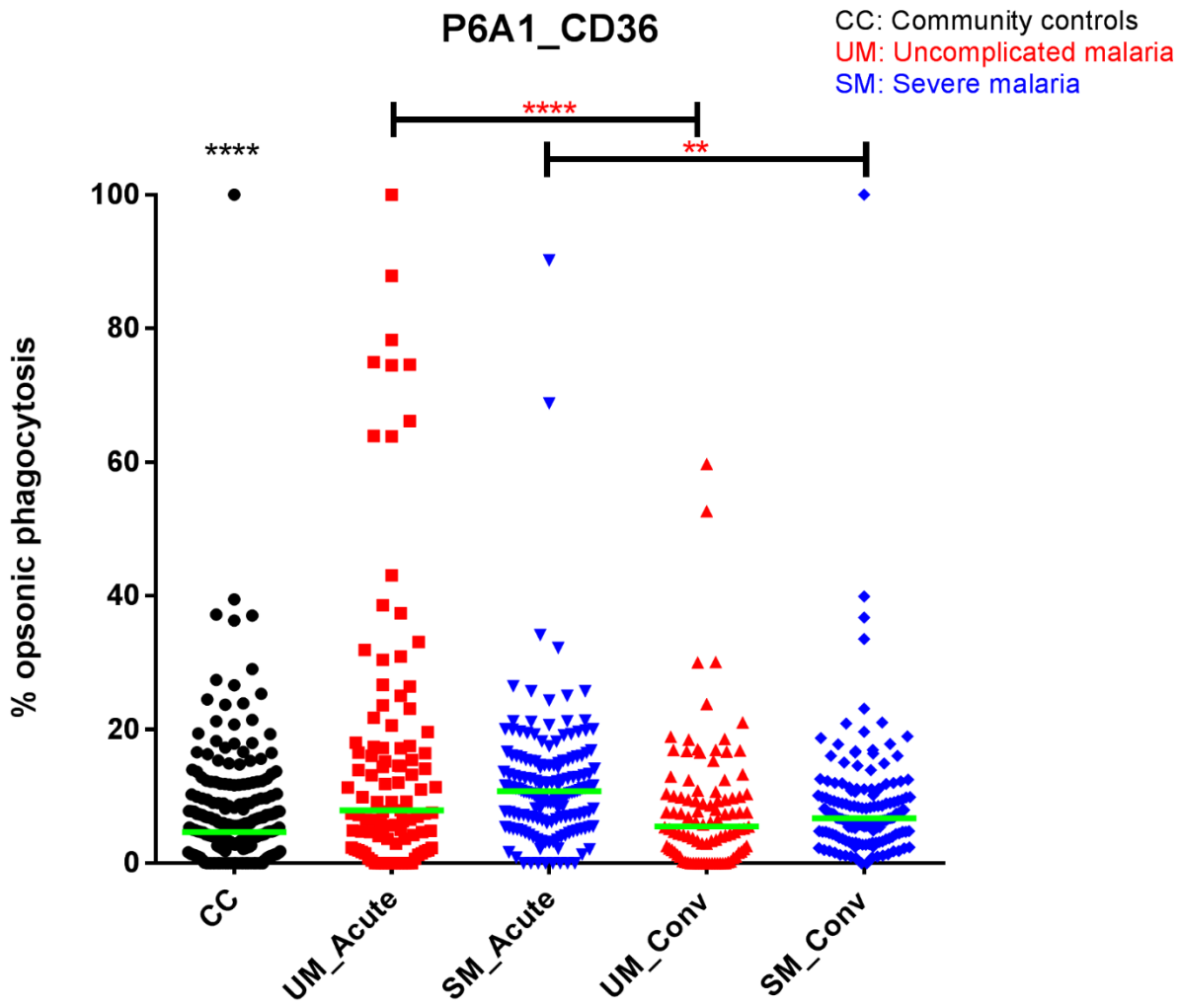


Figure 5.4 Opsonic antibody levels against CD36-binding PfEMP1 (P6A1 parasite line) in acute and convalescent plasma.

Using a Wilcoxon Rank Sum (for unpaired samples) or Wilcoxon matched-pairs signed rank test (for paired samples), antibody levels were compared between children with severe malaria and uncomplicated malaria at acute and convalescent timepoints and community controls. Y-axis represents the percent opsonic phagocytosis, that is, percentage of THP-1 cells that have phagocytosed the infected erythrocyte relative to the positive control. Median antibody levels are presented as green horizontal bars for each disease severity group. Significant associations indicated by p-values: * < 0.05, ** < 0.01, *** <0.001, **** < 0.0001 (black *: unpaired test, red *: paired test). Total number of participants in the severe acute group, n = 163; severe convalescent group, n = 158; uncomplicated acute malaria group, n= 101 and uncomplicated convalescent group, n = 99. CC, community controls; UM, uncomplicated malaria; SM, severe malaria; Conv, convalescent.

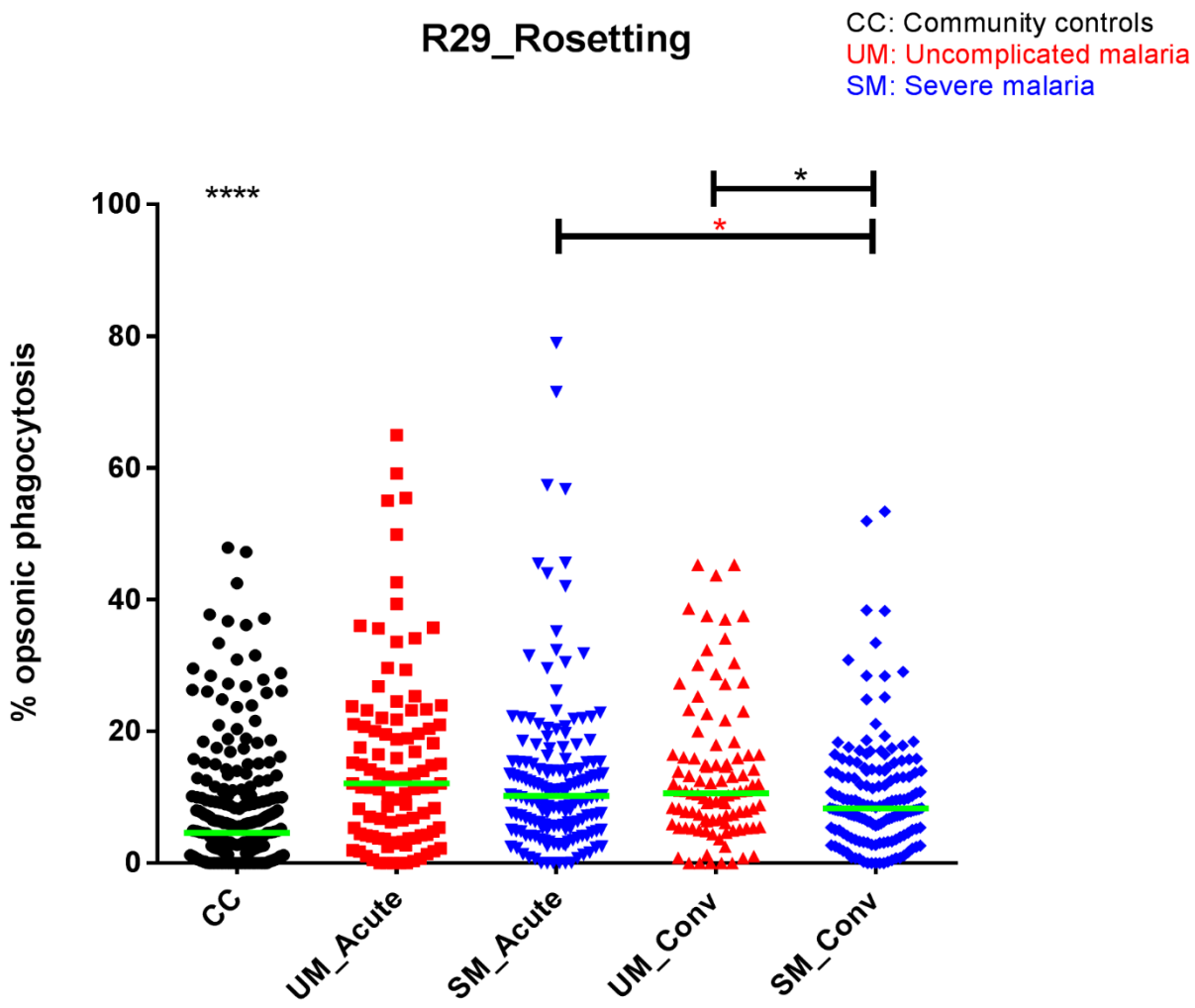


Figure 5.5 Opsonic antibody levels against PfEMP1 associated with rosetting (R29 parasite line) in acute and convalescent plasma.

Using a Wilcoxon Rank Sum (for unpaired samples) or Wilcoxon matched-pairs signed rank test (for paired samples), antibody levels were compared between children with severe malaria and uncomplicated malaria at acute and convalescent timepoints and community controls. Y-axis represents the percent opsonic phagocytosis, that is, percentage of THP-1 cells that have phagocytosed the infected erythrocyte relative to the positive control. Median antibody levels are presented as green horizontal bars for each disease severity group. Significant associations indicated by p-values: * < 0.05, ** < 0.01, *** <0.001, **** < 0.0001 (black *: unpaired test, red *: paired test). Total number of participants in the severe acute group, n = 163; severe convalescent group, n = 158; uncomplicated acute malaria group, n= 101 and uncomplicated convalescent group, n = 99. CC, community controls; UM, uncomplicated malaria; SM, severe malaria; Conv, convalescent.

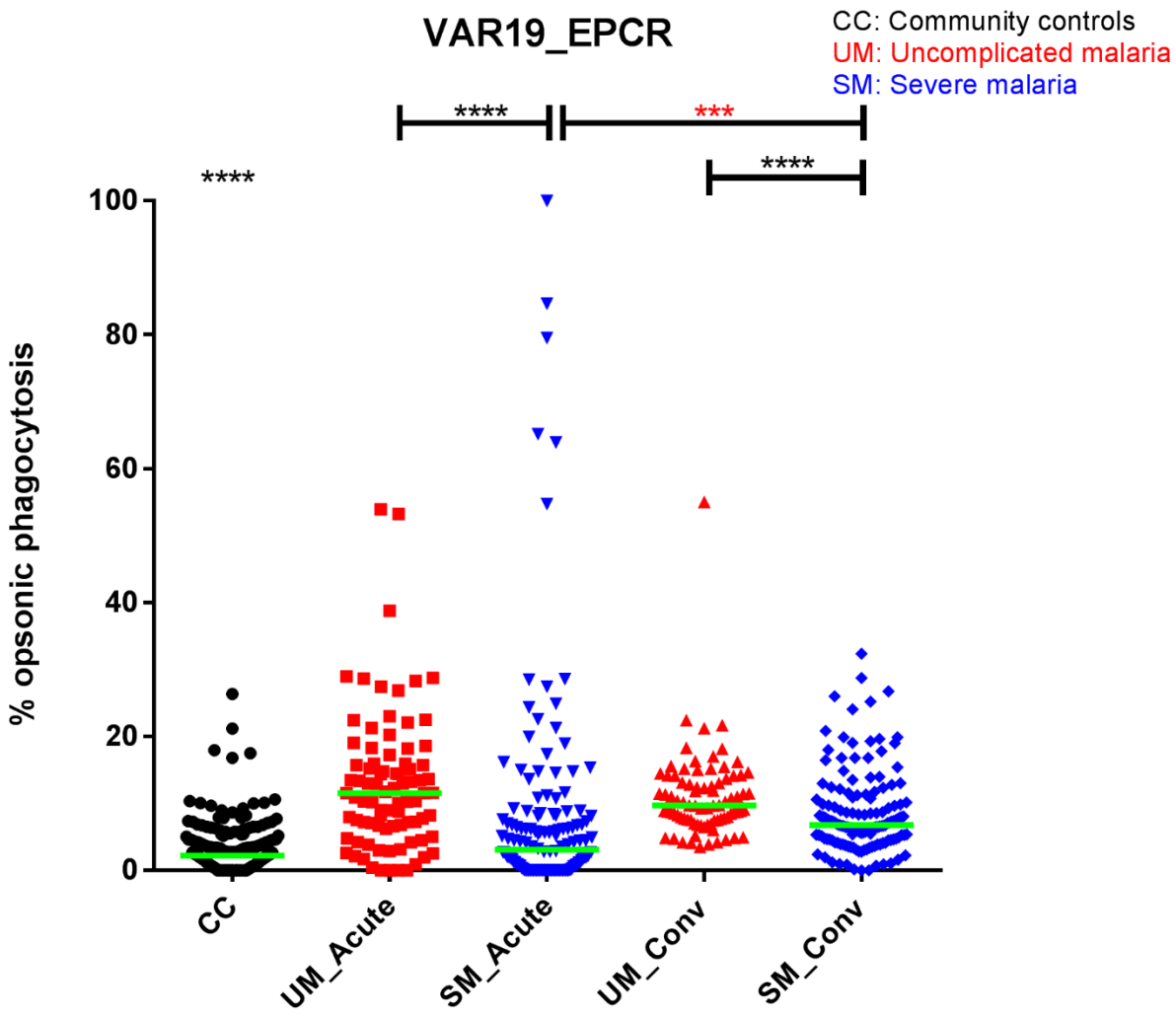


Figure 5.6 Opsonic antibody levels against EPCR-binding PfEMP1 (Var19 parasite line) in acute and convalescent plasma.

Using a Wilcoxon Rank Sum (for unpaired samples) or Wilcoxon matched-pairs signed rank test (for paired samples), antibody levels were compared between children with severe malaria and uncomplicated malaria at acute and convalescent timepoints and community controls. Y-axis represents the percent opsonic phagocytosis, that is, percentage of THP-1 cells that have phagocytosed the infected erythrocyte relative to the positive control. Median antibody levels are presented as green horizontal bars for each disease severity group. Significant associations indicated by p-values: * < 0.05, ** < 0.01, *** <0.001, **** < 0.0001 (black *: unpaired test, red *: paired test). Total number of participants in the severe acute group, n = 163; severe convalescent group, n = 158; uncomplicated acute malaria group, n= 101 and uncomplicated convalescent group, n = 99. CC, community controls; UM, uncomplicated malaria; SM, severe malaria; Conv, convalescent.

5.3.4 Opsonic antibody levels against different PfEMP1 types in children with severe malaria syndromes at presentation and during convalescence

Next, we determined if opsonising antibody levels differed between children with specific severe malaria syndromes and children with uncomplicated malaria (who did not have the severe malaria syndromes (normal)). This could provide insights on the type of PfEMP1 associated with the syndromes and association of these antibodies with protection from the specific syndrome.

The different severe malaria syndromes tested were selected based on the frequency of their occurrence in this cohort and included: severe anaemia; a BCS of 0-4: with a BCS of 0-2 indicative of deep coma and BCS of 3-4 indicative of impaired consciousness; hyperlactataemia; metabolic acidosis and respiratory distress (Table 5.3). Due to limited numbers of children with BCS 0-2 and BCS 3-4, we combined children with BCS 0-4 for our analyses and collectively called them as children with impaired consciousness. Respiratory distress and hyperlactataemia are often considered as surrogates for metabolic acidosis. Once again, due to limited numbers of children with metabolic acidosis and respiratory distress compared to hyperlactataemia, we used hyperlactataemia as a representative of metabolic acidosis to assess its association with opsonising antibodies. The overlapping severe malaria syndromes in these children has been presented in figure 5.1.

The different PfEMP1 types tested were ICAM-1 binders, CD36 binders, PfEMP1 associated with rosetting and EPCR binders.

5.3.4.1 Opsonic antibody levels at <u>presentation</u> in children with severe malarial anaemia and children with uncomplicated malaria

At presentation, opsonic antibody levels in children with severe malarial anaemia were compared to children with uncomplicated malaria who had normal haemoglobin levels against the four PfEMP1 types: ICAM-1 binders, CD36 binders, PfEMP1 associated with rosetting and EPCR binders.

<u>ICAM-1-binding PfEMP1-E8B3X parasite line</u>

The antibody levels were higher in children with severe malarial anaemia than children with uncomplicated malaria at presentation for E8B3X ($p = 0.042$) (Figure 5.7A).

CD36-binding PfEMP1-P6A1 parasite line

At hospital presentation, there was no difference in the opsonic antibody levels between children with severe malarial anaemia and children with uncomplicated malaria for P6A1 ($p = 0.241$) (Figure 5.7B).

PfEMP1 associated with rosetting- R29 parasite line

Opsonic antibody levels in acute plasma were similar for children with severe malarial anaemia and children with uncomplicated malaria for R29 ($p = 0.912$) (Figure 5.7C).

EPCR-binding PfEMP1- Var19 parasite line

No significant difference in the opsonic antibody levels was found on comparing children with severe malarial anaemia and children with uncomplicated malaria at presentation for Var19 line ($p = 0.079$) (Figure 5.7D).

5.3.4.2 Opsonic antibody levels during convalescence in children with severe malarial anaemia and children with uncomplicated malaria

Plasma samples collected during convalescence were used to measure differences in opsonic antibody levels in children with severe malarial anaemia and children with uncomplicated malaria with normal haemoglobin levels against the four PfEMP1 types.

ICAM-1-binding PfEMP1-E8B3X parasite line

The opsonic antibody levels were similar in convalescent children with severe malarial anaemia and children convalescent from uncomplicated malaria against the E8B3X line ($p = 0.922$) (Figure 5.8A).

CD36-binding PfEMP1-P6A1 parasite line

On comparing the opsonic antibody levels between children with severe malarial anaemia and children with uncomplicated malaria at convalescence, no significant difference was observed for P6A1 ($p = 0.115$) (Figure 5.8B).

PfEMP1 associated with rosetting- R29 parasite line

At convalescence, when opsonic antibody levels against R29 were compared between children with severe malarial anaemia and uncomplicated malaria, children with severe anaemia had lower antibody levels than those with uncomplicated malaria ($p = 0.029$) (Figure 5.8C).

EPCR-binding PfEMP1- Var19 parasite line

Children with severe malarial anaemia had lower opsonic antibody levels as compared to children with uncomplicated malaria during convalescence for Var19 ($p = 0.040$) (Figure 5.8D)

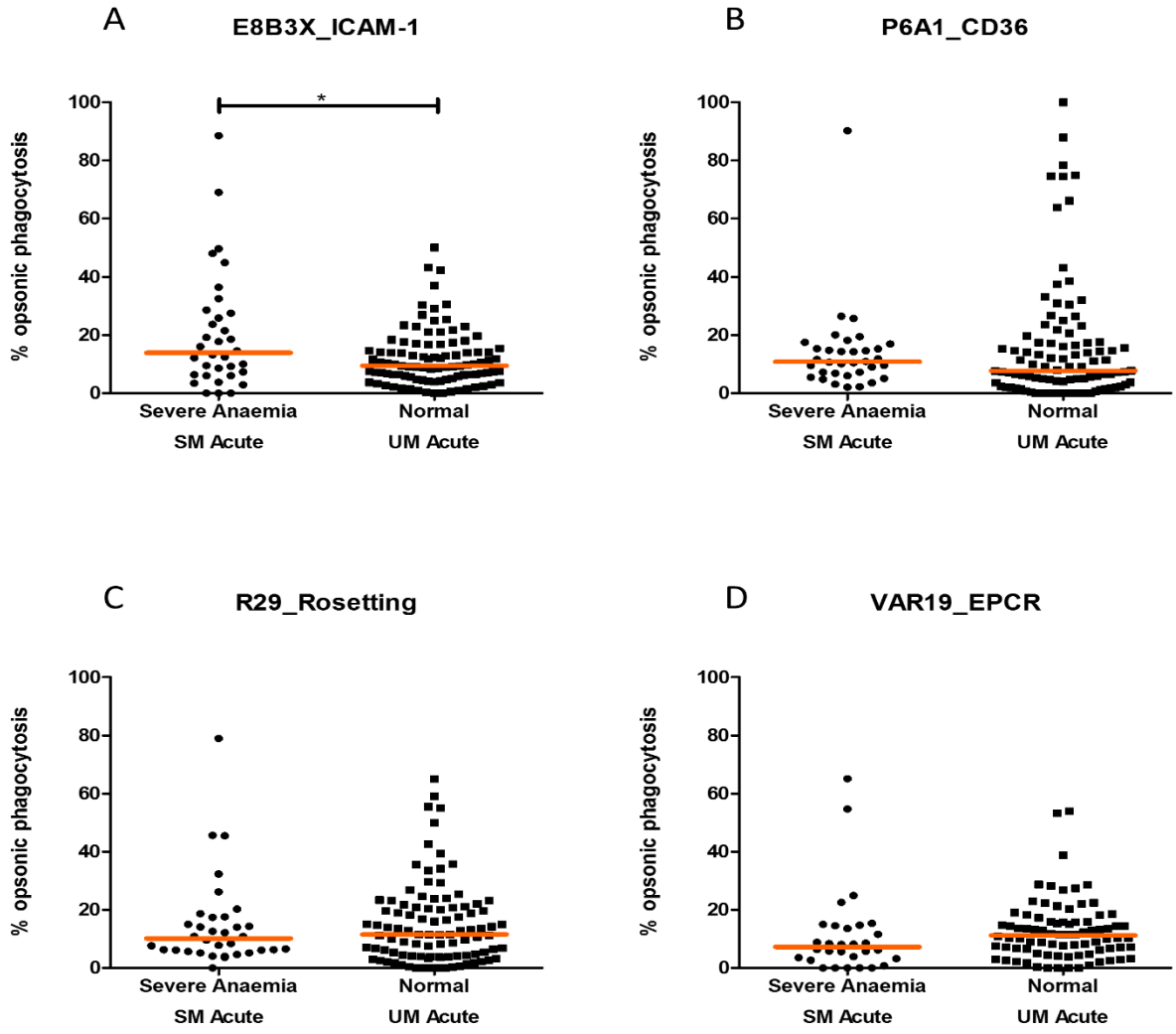


Figure 5.7 Opsonic antibody levels at <u>presentation</u> in children with severe malarial anaemia and children with uncomplicated malaria.

Using a Wilcoxon Rank Sum test, antibody levels were compared between children with severe malarial anaemia and uncomplicated malaria at acute timepoint. Y-axis represents the percent opsonic phagocytosis, that is, percentage of THP-1 cells that have phagocytosed the infected erythrocyte relative to the positive control. Median antibody levels are presented as orange horizontal bars for each disease severity group. Significant associations indicated by p-values: * < 0.05, ** < 0.01, *** <0.001, **** < 0.0001. Total number of participants in the acute severe malarial anaemia group, n = 34 (30 for Var19) and uncomplicated acute malaria group, n= 97 (82 for Var19). Opsonic antibody levels in the different disease severity groups were compared against four PfEMP1 types: A. E8B3X, binding ICAM-1. B. P6A1, binding CD36. C. R29, associated with rosetting. D. VAR19, binding EPCR. SM, severe malaria; UM, uncomplicated malaria.

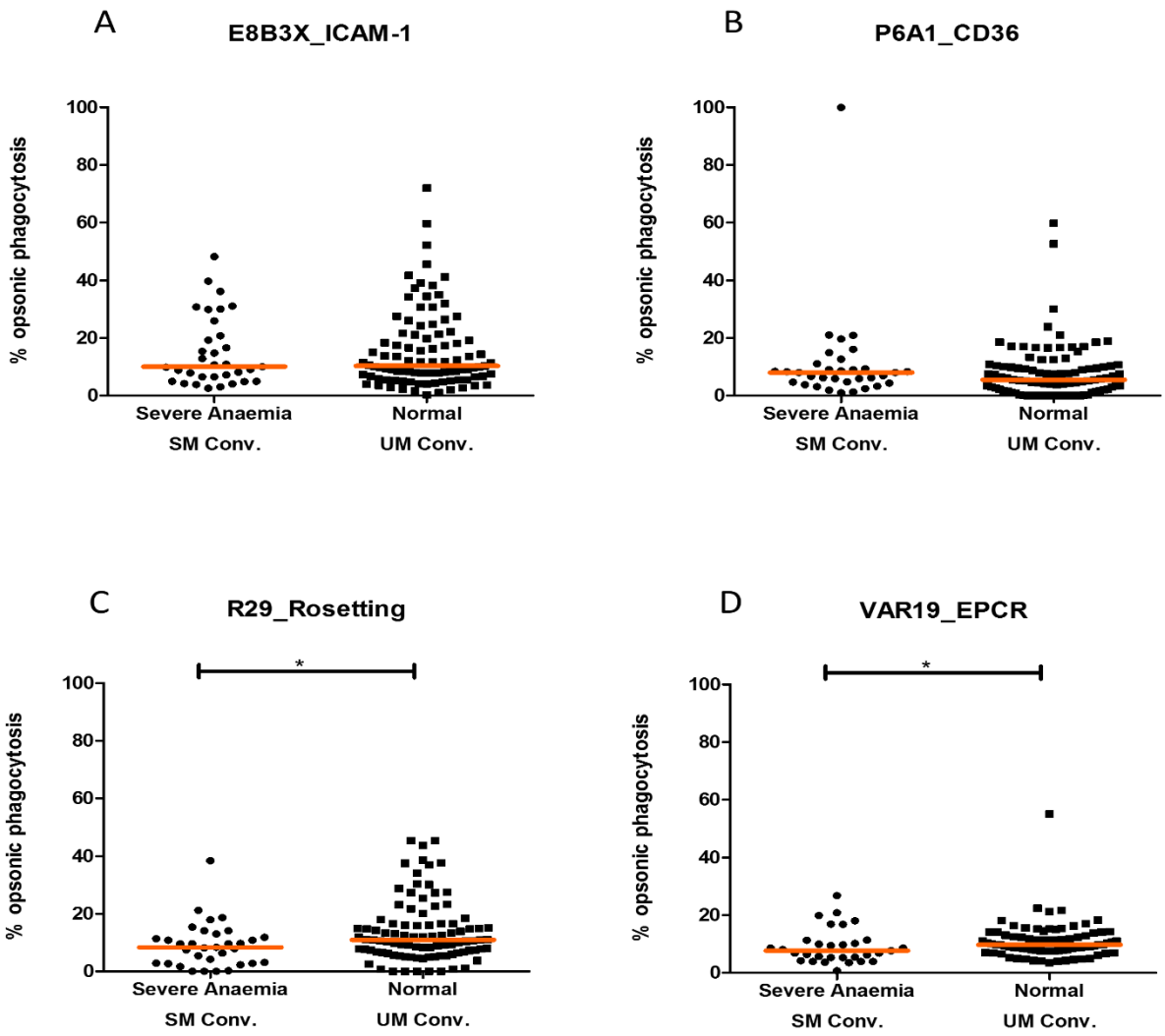


Figure 5.8 Opsonic antibody levels during <u>convalescence</u> in children with severe malarial anaemia and children with uncomplicated malaria.

Using a Wilcoxon Rank Sum test, antibody levels were compared between children with severe malarial anaemia and uncomplicated malaria during convalescence. Y-axis represents the percent opsonic phagocytosis, that is, percentage of THP-1 cells that have phagocytosed the infected erythrocyte relative to the positive control. Median antibody levels are presented as orange horizontal bars for each disease severity group. Significant associations indicated by p-values: * < 0.05, ** < 0.01, *** <0.001, **** < 0.0001. Total number of participants in the convalescent severe malarial anaemia group, n = 33 (31 for Var19) and uncomplicated convalescent malaria group, n= 95. Opsonic antibody levels in the different disease severity groups were compared against four PfEMP1 types: A. E8B3X, binding ICAM-1. B. P6A1, binding CD36. C. R29, associated with rosetting. D. VAR19, binding EPCR. SM, severe malaria; Conv, convalescent; UM, uncomplicated malaria.

5.3.4.3 Opsonic antibody levels at presentation in children with impaired consciousness and children with uncomplicated malaria.

At presentation, opsonic antibody levels in children with impaired consciousness (BCS 0-4) were compared to children with uncomplicated malaria who had normal consciousness (BCS 5) against four PfEMP1 types: ICAM-1 binders, CD36 binders, PfEMP1 associated with rosetting and EPCR binders.

ICAM-1-binding PfEMP1-E8B3X parasite line

At hospital presentation, children with impaired consciousness had significantly higher levels of opsonic antibodies when compared to children with uncomplicated malaria for the E8B3X line ($z = 3.5$, $p = 0.0005$) (Figure 5.9A).

CD36-binding PfEMP1-P6A1 parasite line

When opsonic antibody levels were compared between children with impaired consciousness and children with uncomplicated malaria against P6A1 at presentation, no significant difference in the antibody levels was observed. ($z = 1.9$, $p = 0.062$) (Figure 5.9B).

PfEMP1 associated with rosetting- R29 parasite line

For R29, no difference in the opsonic antibody levels was observed at presentation, when children with impaired consciousness were compared to children with uncomplicated malaria ($z = 0.2$, $p = 0.824$) (Figure 5.9C).

EPCR-binding PfEMP1- Var19 parasite line

Opsonic antibody levels at hospital presentation for Var19 were significantly higher in children with uncomplicated malaria than children with impaired consciousness ($z = 4.2$, $p < 0.0001$) (Figure 5.9D).

5.3.4.4 Opsonic antibody levels during convalescence in children with impaired consciousness and children with uncomplicated malaria.

Opsonic antibodies in children convalescent from severe malaria who had impaired consciousness (BCS of 0-4) were compared to children convalescent from uncomplicated malaria (who had normal consciousness, BCS 5) against PfEMP1 binding ICAM-1, CD36, associated with rosetting and binding EPCR.

ICAM-1-binding PfEMP1-E8B3X parasite line

During convalescence, the opsonic antibody levels in children with impaired consciousness and children with uncomplicated malaria were broadly similar for E8B3X ($z = -1.2$, $p = 0.221$) (Figure 5.10A).

CD36-binding PfEMP1-P6A1 parasite line

No significant difference was observed on comparing the opsonic antibody levels in children with impaired consciousness and children with uncomplicated malaria during convalescence against P6A1 line ($z = 0.8$, $p = 0.433$) (Figure 5.10B).

PfEMP1 associated with rosetting- R29 parasite line

Children convalescent from severe malaria having impaired consciousness showed a weak evidence for having lower levels of opsonic antibodies to R29 when compared to children convalescent from uncomplicated malaria ($z = -2.0$, $p = 0.049$) (Figure 5.10C).

EPCR-binding PfEMP1- Var19 parasite line

Opsonic antibody levels were lower in children with impaired consciousness than children with uncomplicated malaria during convalescence for the Var19 line ($z = -2.2$, $p = 0.028$) (Figure 5.10D).

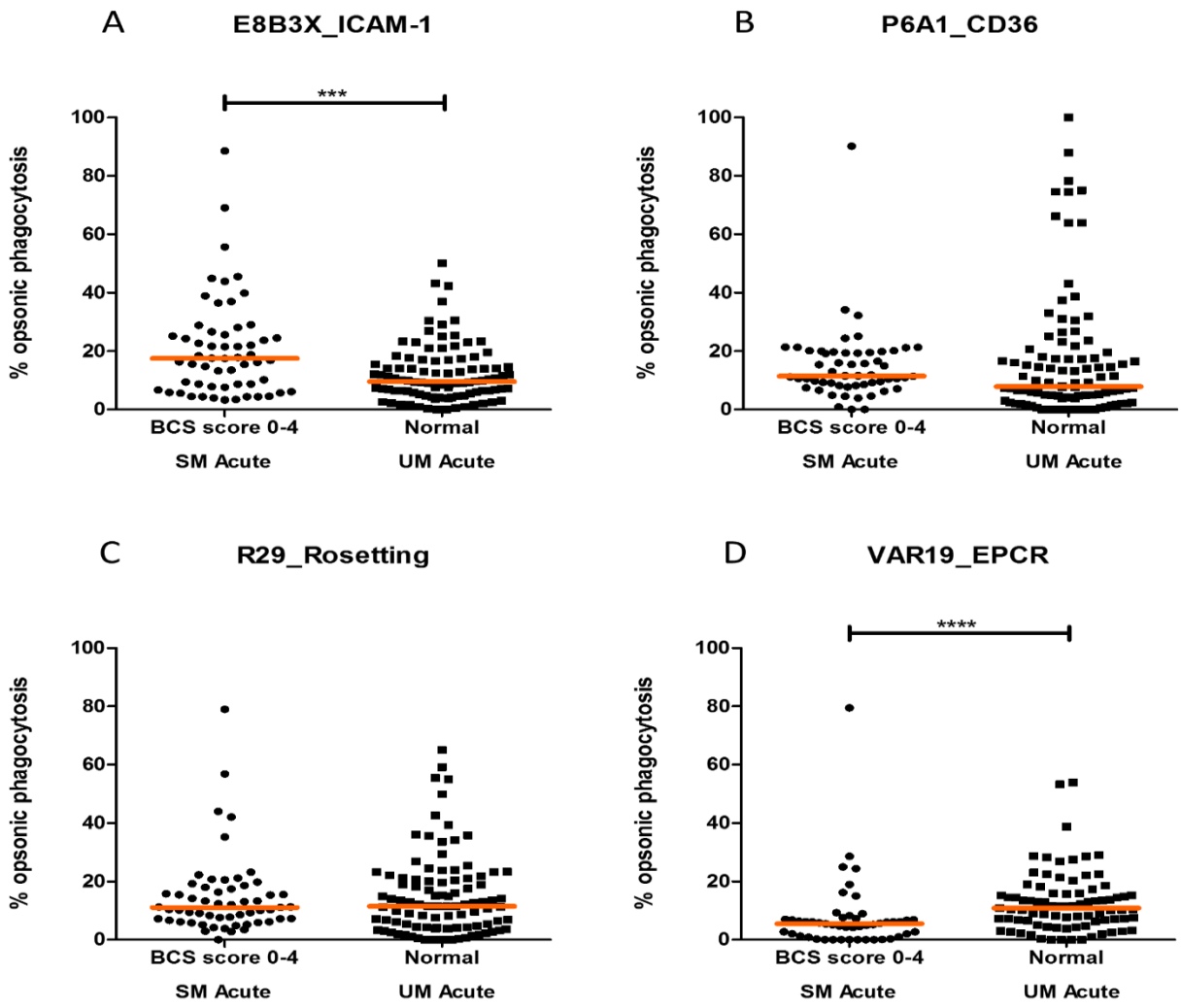


Figure 5.9 Opsonic antibody levels at <u>presentation</u> in children with impaired consciousness (BCS 0-4) and children with uncomplicated malaria.

Using a Wilcoxon Rank Sum test, antibody levels were compared between children with impaired consciousness and uncomplicated malaria at acute timepoint. Y-axis represents the percent opsonic phagocytosis, that is, percentage of THP-1 cells that have phagocytosed the infected erythrocyte relative to the positive control. Median antibody levels are presented as orange horizontal bars for each disease severity group. Significant associations indicated by p-values: * < 0.05, ** < 0.01, *** <0.001, **** < 0.0001. Total number of participants in the acute group with impaired consciousness, n = 57 (49 for Var19) and uncomplicated acute malaria group, n= 98 (83 for Var19). Opsonic antibody levels in the different disease severity groups were compared against four PfEMP1 types: A. E8B3X, binding ICAM-1. B. P6A1, binding CD36. C. R29, associated with rosetting. D. VAR19, binding EPCR. SM, severe malaria; UM, uncomplicated malaria.

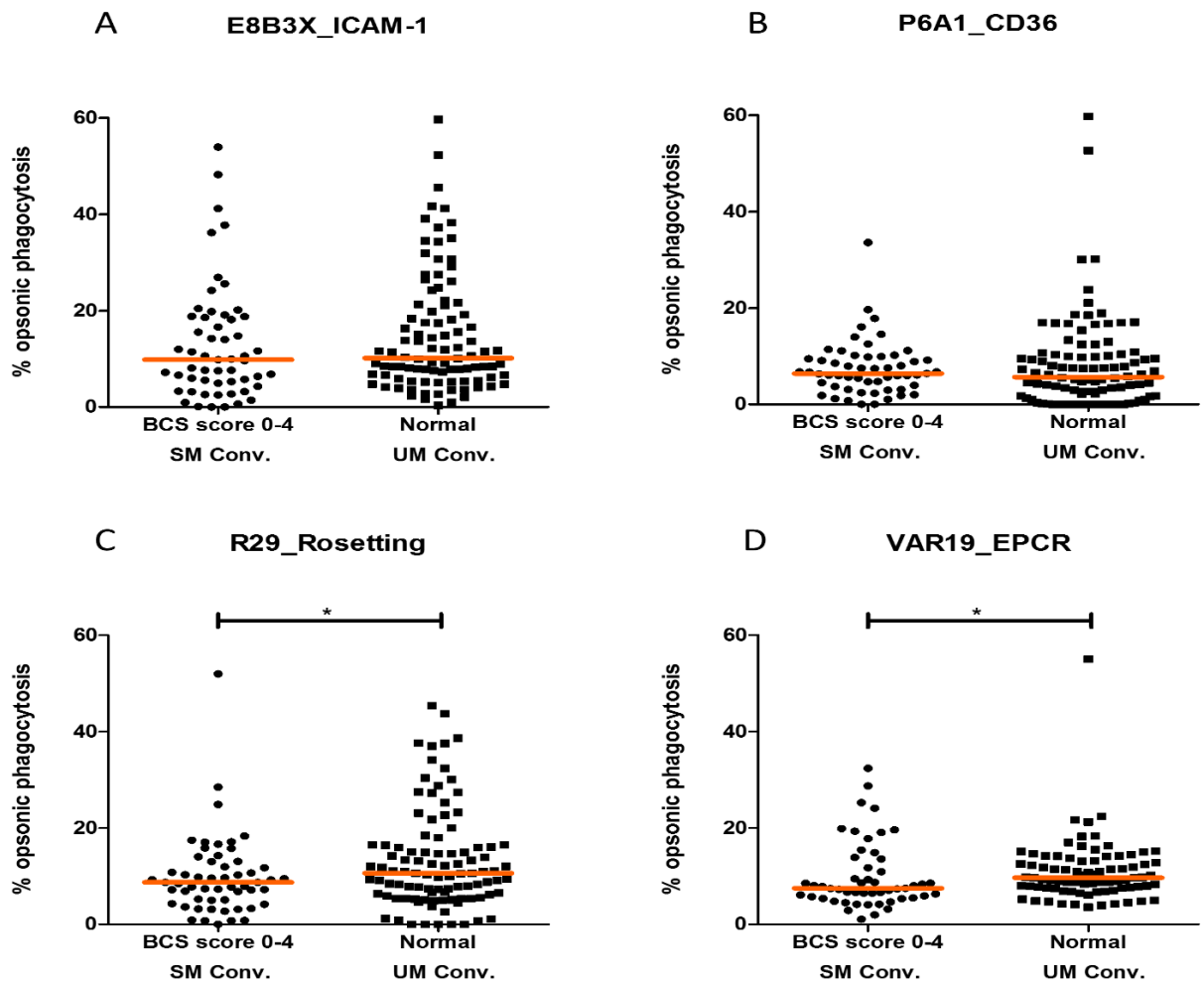


Figure 5.10 Opsonic antibody levels during <u>convalescence</u> in children with impaired consciousness (BCS 0-4) and children with uncomplicated malaria.

Using a Wilcoxon Rank Sum test, antibody levels were compared between children with impaired consciousness and uncomplicated malaria during convalescence. Y-axis represents the percent opsonic phagocytosis, that is, percentage of THP-1 cells that have phagocytosed the infected erythrocyte relative to the positive control. Median antibody levels are presented as orange horizontal bars for each disease severity group. Significant associations indicated by p-values: * < 0.05, ** < 0.01, *** <0.001, **** < 0.0001. Total number of participants in the convalescent group with impaired consciousness, n = 55 (49 for Var19) and uncomplicated convalescent malaria group, n= 98 (83 for Var19). Opsonic antibody levels in the different disease severity groups were compared against four PfEMP1 types: A. E8B3X, binding ICAM-1. B. P6A1, binding CD36. C. R29, associated with rosetting. D. VAR19, binding EPCR. SM, severe malaria; Conv, convalescent; UM, uncomplicated malaria.

5.3.4.5 Opsonic antibody levels at presentation in children with severe malaria with hyperlactataemia and children with uncomplicated malaria

At presentation, children who had severe malaria along with hyperlactataemia (blood lactate > 5 mmol/L) were compared to children with uncomplicated malaria who had normal blood lactate levels for opsonising antibodies against the four PfEMP1 types: ICAM-1 binders, CD36 binders, associated with rosetting, EPCR binders.

ICAM-1-binding PfEMP1-E8B3X parasite line

On comparing opsonic antibody levels against E8B3X at presentation in children with hyperlactataemia to children with uncomplicated malaria, no significant difference in the antibody levels was observed ($p = 0.078$) (Figure 5.11A).

CD36-binding PfEMP1-P6A1 parasite line

Opsonic antibody levels did not vary significantly between children with hyperlactataemia and children with uncomplicated malaria at presentation for P6A1 ($p = 0.261$) (Figure 5.11B).

PfEMP1 associated with rosetting- R29 parasite line

At presentation, the opsonic antibody levels were similar between children hyperlactataemia and children with uncomplicated malaria for R29 ($p = 0.634$) (Figure 5.11C).

EPCR-binding PfEMP1- Var19 parasite line

Children with hyperlactataemia had significantly lower opsonic antibody levels against Var19 when compared to children with uncomplicated malaria at hospital presentation ($p = 0.0007$) (Figure 5.11D).

5.3.4.6 Opsonic antibody levels during convalescence in children with hyperlactataemia and children with uncomplicated malaria

Opsonic antibodies in children convalescent from severe malaria with hyperlactataemia (blood lactate > 5 mmol/L) were compared to children convalescent from uncomplicated malaria against PfEMP1 binding ICAM-1, CD36, associated with rosetting and binding EPCR.

ICAM-1-binding PfEMP1-E8B3X parasite line

Opsonic antibody levels in children convalescent from severe malaria with hyperlactataemia were similar to children convalescent from uncomplicated malaria for E8B3X line ($p = 0.186$) (Figure 5.12A).

CD36-binding PfEMP1-P6A1 parasite line

During convalescence, children with hyperlactataemia and children with uncomplicated malaria had similar levels of opsonising antibodies against P6A1 ($p = 0.619$) (Figure 5.12B).

PfEMP1 associated with rosetting- R29 parasite line

Children convalescent from severe malaria with hyperlactataemia had lower levels of opsonic antibodies to R29 than children with uncomplicated malaria ($p = 0.025$) (Figure 5.12C).

EPCR-binding PfEMP1- Var19 parasite line

Opsonic antibody levels were significantly lower in children with severe malaria with hyperlactataemia as compared to children with uncomplicated malaria during convalescence for Var19 line ($p = 0.002$) (Figure 5.12D).

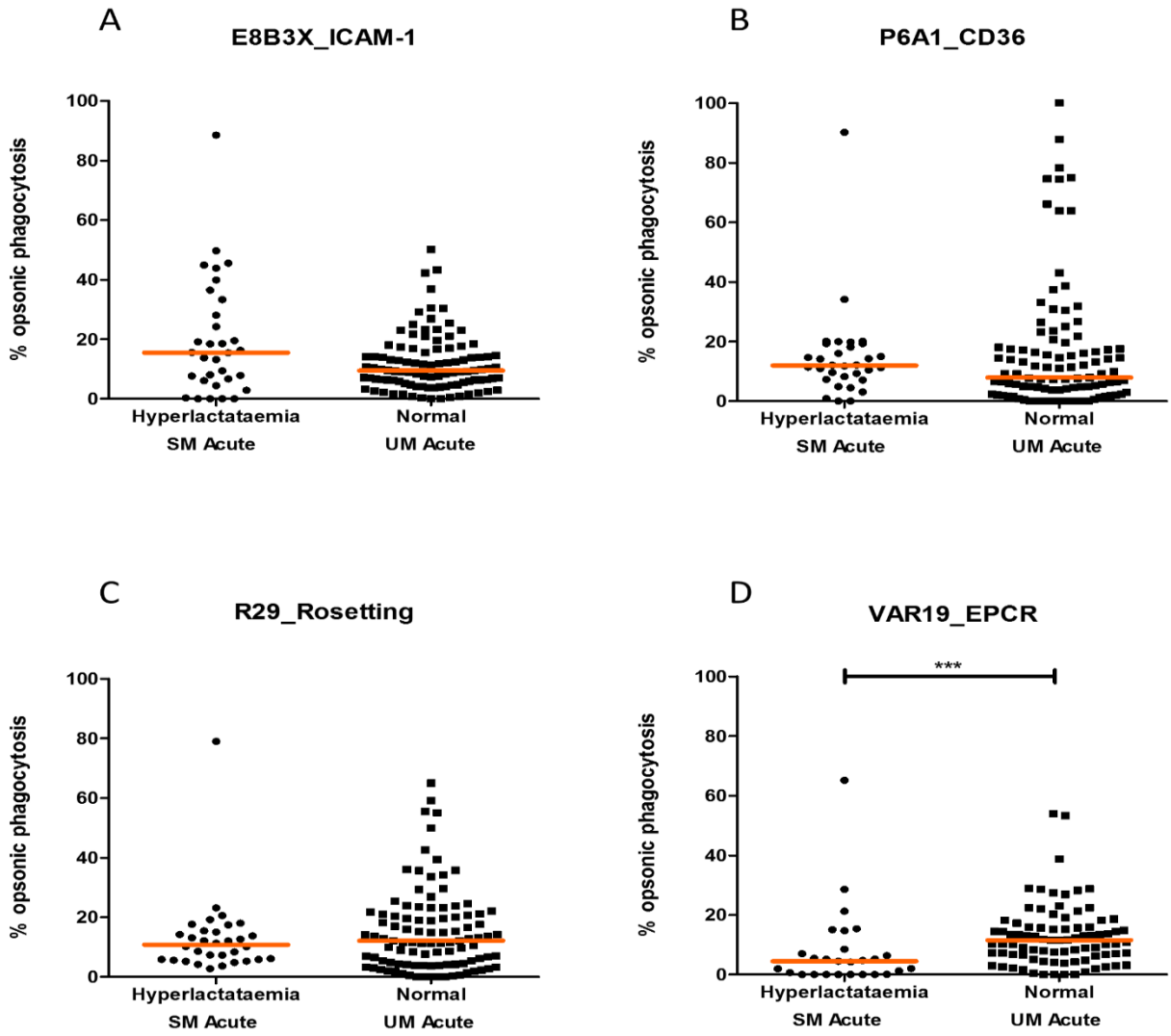


Figure 5.11 Opsonic antibody levels at <u>presentation</u> in children with hyperlactataemia and children with uncomplicated malaria.

Using a Wilcoxon Rank Sum test, antibody levels were compared between children with hyperlactataemia and uncomplicated malaria at acute timepoint. Y-axis represents the percent opsonic phagocytosis, that is, percentage of THP-1 cells that have phagocytosed the infected erythrocyte relative to the positive control. Median antibody levels are presented as orange horizontal bars for each disease severity group. Significant associations indicated by p-values: * < 0.05, ** < 0.01, *** <0.001, **** < 0.0001. Total number of participants in the acute group with hyperlactataemia, n = 32 (27 for Var19) and uncomplicated acute malaria group, n= 101 (85 for Var19). Opsonic antibody levels in the different disease severity groups were compared against four PfEMP1 types: A. E8B3X, binding ICAM-1. B. P6A1, binding CD36. C. R29, associated with rosetting. D. VAR19, binding EPCR. SM, severe malaria; UM, uncomplicated malaria.

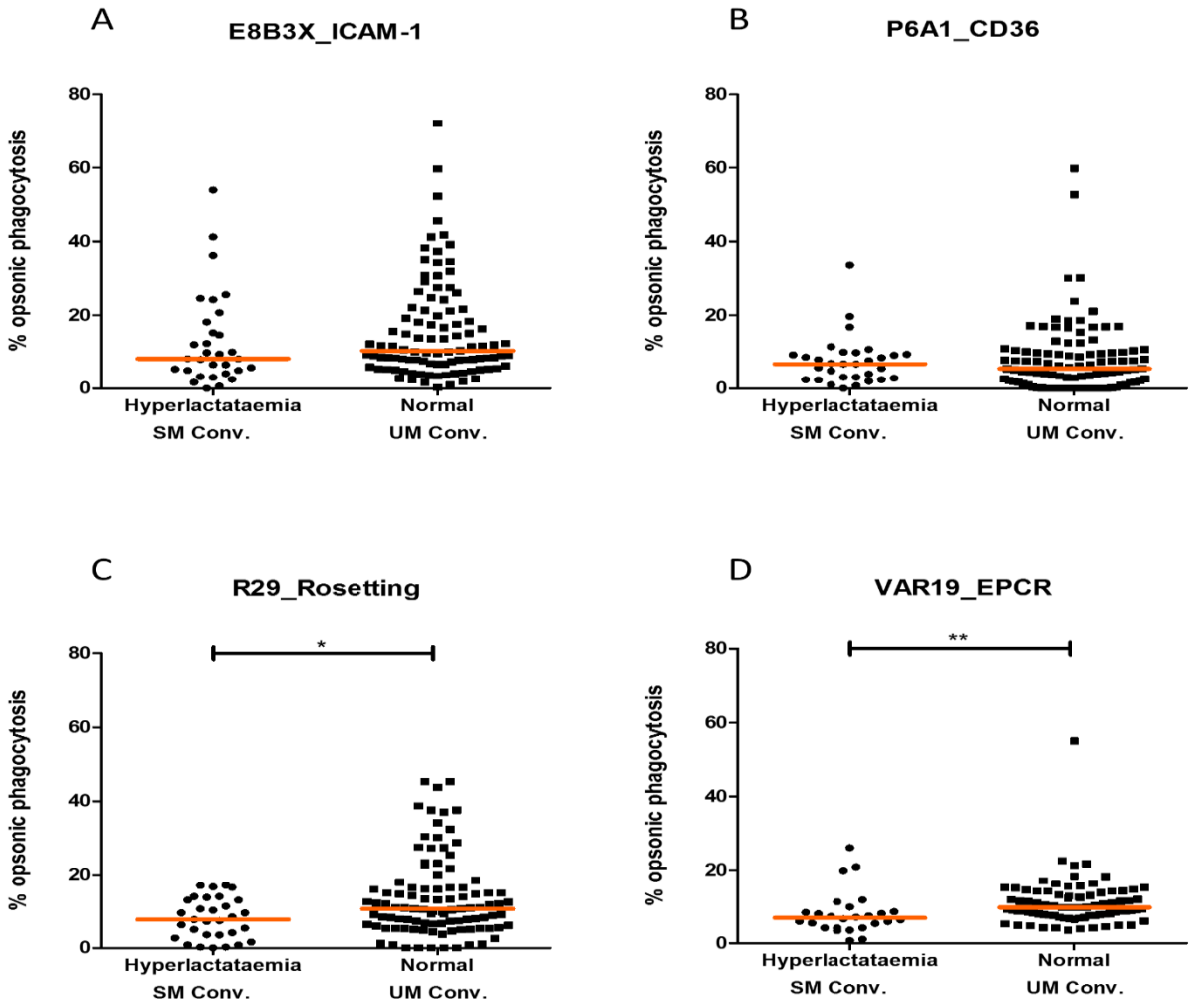


Figure 5.12 Opsonic antibody levels during convalescence in children with hyperlactataemia and children with uncomplicated malaria.

Using a Wilcoxon Rank Sum test, antibody levels were compared between children with hyperlactataemia and uncomplicated malaria during convalescence. Y-axis represents the percent opsonic phagocytosis, that is, percentage of THP-1 cells that have phagocytosed the infected erythrocyte relative to the positive control. Median antibody levels are presented as orange horizontal bars for each disease severity group. Significant associations indicated by p-values: * < 0.05, ** < 0.01, *** <0.001, **** < 0.0001. Total number of participants in the convalescent group with hyperlactataemia, n = 31 (26 for Var19) and uncomplicated convalescent malaria group, n= 99 (82 for Var19). Opsonic antibody levels in the different disease severity groups were compared against four PfEMP1 types: A. E8B3X, binding ICAM-1. B. P6A1, binding CD36. C. R29, associated with rosetting. D. VAR19, binding EPCR. SM, severe malaria; Conv, convalescent; UM, uncomplicated malaria.

5.3.5 Opsonic antibody levels in children with severe malarial anaemia or impaired consciousness or hyperlactataemia

Having determined the differences in opsonising antibody levels for children with a particular severe syndrome versus children with uncomplicated malaria, we next determined if the antibody levels differed in children with severe malaria who had either severe malarial anaemia or impaired consciousness (BCS 0-4) or hyperlactataemia (blood lactate > 5 mmol/L). That is, we determined if antibody levels differed based on type of severe malaria syndrome presented by the children. Opsonic antibody levels were compared between children with either of these syndromes against the four PfEMP1 types: ICAM-1 binders, CD36 binders, associated with rosetting or EPCR binders. These antibody level comparisons were made in acute and convalescent plasma.

5.3.5.1 Opsonic antibody levels in children with severe malarial anaemia or impaired consciousness or hyperlactataemia at <u>presentation</u>

At presentation, when all three syndromes were compared, that is, children with severe malarial anaemia or impaired consciousness or hyperlactataemia were compared, opsonic antibody levels against ICAM-1 binders, CD36 binders, PfEMP1 associated with rosetting or EPCR binders did not differ significantly ($p = 0.743$, 0.594, 0.785 and 0.206 respectively). Similarly, antibody levels did not differ when children with severe malarial anaemia were compared to children with impaired consciousness or hyperlactataemia, or when children with impaired consciousness were compared to children with hyperlactataemia for either of the PfEMP1 types tested. Results are presented in table 5.4 and figure 5.13.

5.3.5.2 Opsonic antibody levels in children with severe malarial anaemia or impaired consciousness or hyperlactataemia during <u>convalescence</u>

During convalescence, when all three syndromes were compared, that is, children with severe malarial anaemia or impaired consciousness or hyperlactataemia were compared, opsonic antibody levels against ICAM-1 binders, CD36 binders, PfEMP1 associated with rosetting or EPCR binders did not differ significantly ($p = 0.539$, 0.579, 0.766 and 0.520 respectively). Opsonic antibody levels measured during convalescence and compared between children with severe malarial anaemia and children with impaired consciousness or children with severe malarial anaemia and children with hyperlactataemia or children with impaired consciousness and children with hyperlactataemia against the four PfEMP1 types did not show significant differences. Results are presented in table 5.5 and figure 5.14.

Table 5.4 Comparison of opsonic antibody levels in children with severe malarial anaemia or impaired consciousness or hyperlactataemia at presentation

IE line	Sev anaemia	BCS 0-4	Hyperlact.	Sev Anaemia vs BCS 0-4	Sev. Anaemia vs hyperlact.	BCS 0-4 vs hyperlact.
	Median [IQR]	Median [IQR]	Median [IQR]	P-value	P-value	P-value
E8B3X	13.9 [6.3-27.8]	17.5 [7.8-26.1]	15.5 [6.2-31.9]	0.542	0.947	0.517
P6A1	10.8 [6.7-15.7]	11.5 [8.4-19.6]	11.9 [7.5-19.0]	0.298	0.617	0.723
R29	10.2 [6.1-17.5]	11.1 [6.9-17.7]	10.8 [5.8-15.4]	0.611	0.937	0.533
Var19	7.3 [3.1-14.6]	5.5 [1.1-7.2]	4.5 [0.0-8.5]	0.163	0.129	0.421

E8B3X, binds ICAM-1; P6A1 binds CD36; R29 is a rosetting line, Var19 binds EPCR

Wilcoxon Rank Sum test was used to compare opsonic antibody levels between two severe syndromes.

IE, infected erythrocyte; Sev, severe; BCS, Blantyre coma score; hyperlact, hyperlactataemia

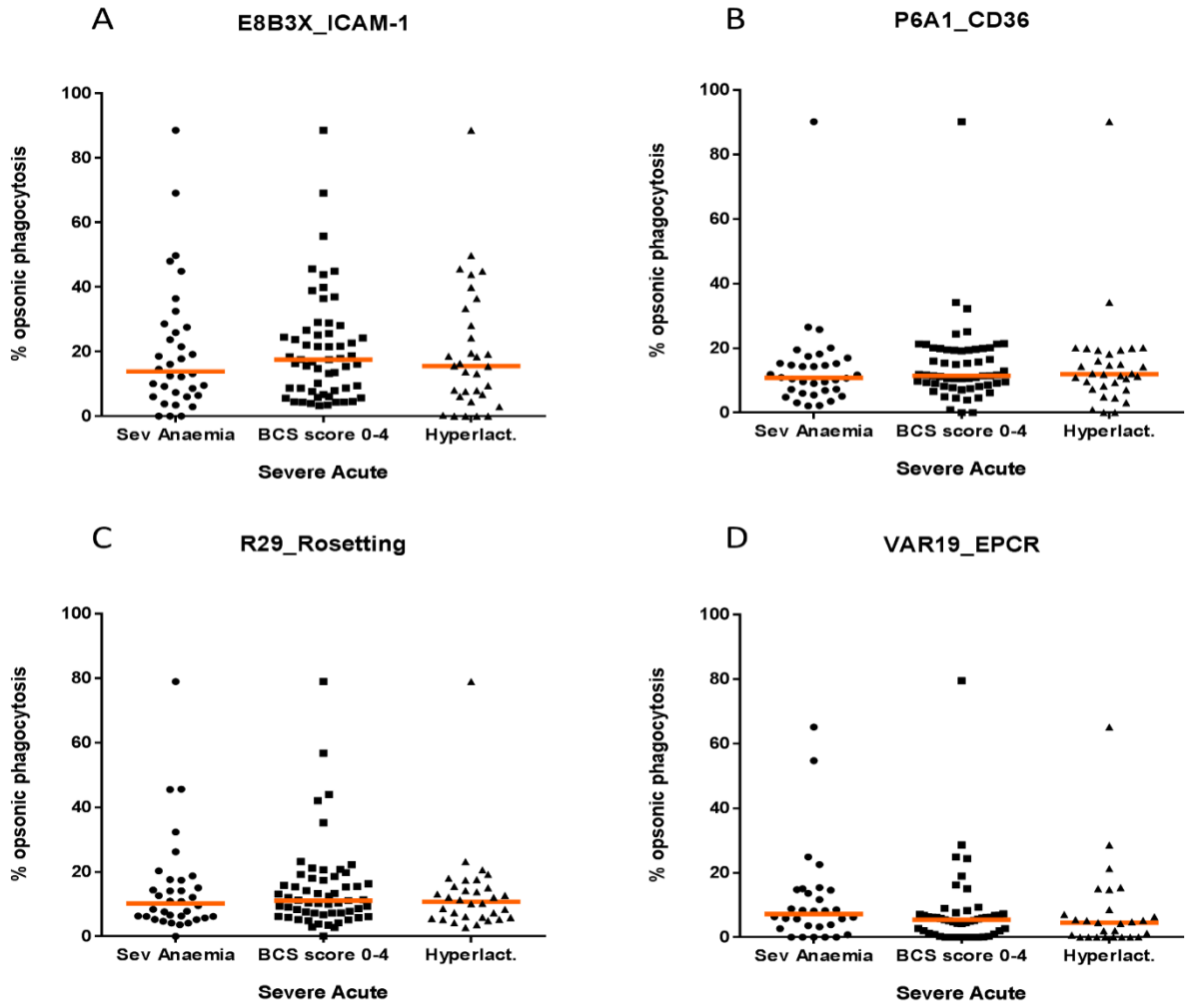


Figure 5.13 Opsonic antibody levels in children with severe malarial anaemia or impaired consciousness or hyperlactataemia at <u>presentation</u>.

Using a Kruskal Wallis test, antibody levels in children with severe malarial anaemia, impaired consciousness (BCS0-4) or hyperlactataemia were compared at presentation. Using a Wilcoxon Rank Sum test, antibody levels were compared between two severe syndromes. Y-axis represents the percent opsonic phagocytosis, that is, percentage of THP-1 cells that have phagocytosed the infected erythrocyte relative to the positive control. Median antibody levels are presented as orange horizontal bars for each disease severity group. Total number of participants in the acute group with severe malarial anaemia, n = 34 (30 for Var19); impaired consciousness, n = 57 (49 for Var19); hyperlactataemia, n = 32 (27 for Var19). Opsonic antibody levels in the different disease severity groups were compared against four PfEMP1 types: A. E8B3X, binding ICAM-1. B. P6A1, binding CD36. C. R29, associated with rosetting. D. VAR19, binding EPCR. Sev, severe; BCS, Blantyre coma score; hyperlact, hyperlactataemia.

Table 5.5 Comparison of opsonic antibody levels in children with severe malarial anaemia or impaired consciousness or hyperlactataemia during convalescence

IE line	Sev anaemia	BCS 0-4	Hyperlact.	Sev Anaemia vs BCS 0-4	Sev. Anaemia vs hyperlact.	BCS 0-4 vs hyperlact.
	Median [IQR]	Median [IQR]	Median [IQR]	P-value	P-value	P-value
E8B3X	10.1 [5.7-23.4]	9.8 [4.9-18.8]	8.2 [4.9-18.2]	0.359	0.305	0.856
P6A1	7.9 [4.5-10.2]	6.4 [3.7-9.8]	6.7 [2.8-9.3]	0.364	0.365	0.863
R29	8.4 [2.8-12.4]	8.7 [5.0-13.0]	7.7 [3.6-13.1]	0.620	0.875	0.501
Var19	7.7 [5.3-11.3]	7.5 [5.5-13.1]	6.8 [4.2-8.9]	0.724	0.496	0.251

E8B3X, binds ICAM-1; P6A1 binds CD36; R29 is a rosetting line, Var19 binds EPCR

Wilcoxon Rank Sum test was used to compare opsonic antibody levels between two severe syndromes.

IE, infected erythrocyte; Sev, severe; BCS, Blantyre coma score; hyperlact, hyperlactataemia.

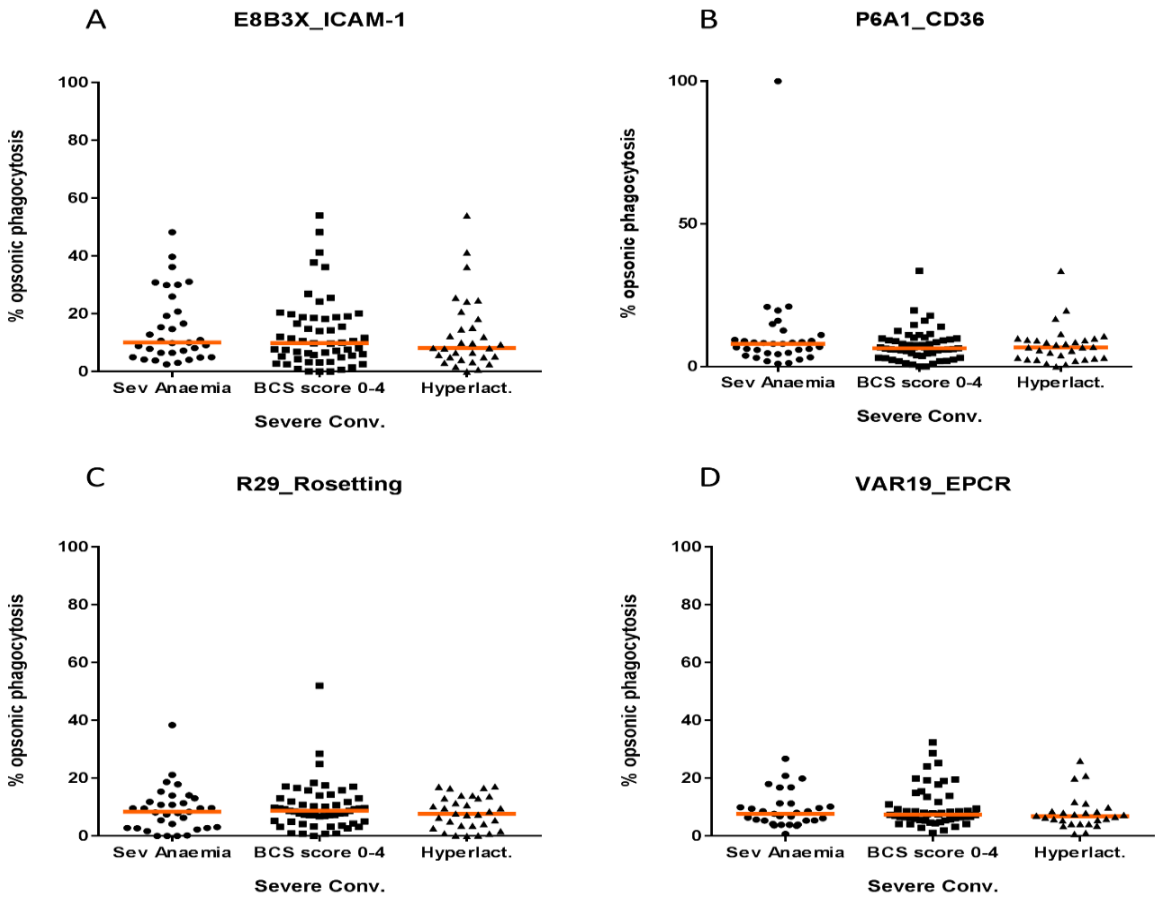


Figure 5.14 Opsonic antibody levels in children with severe malarial anaemia or impaired consciousness or hyperlactataemia during convalescence.

Using a Kruskal Wallis test, antibody levels in children with severe malarial anaemia, impaired consciousness (BCS0-4) or hyperlactataemia were compared during convalescence. Using a Wilcoxon Rank Sum test, antibody levels during convalescence were compared between two severe syndromes. The syndromes compared were severe malarial anaemia, impaired consciousness (BCS0-4) or hyperlactataemia. Y-axis represents the percent opsonic phagocytosis, that is, percentage of THP-1 cells that have phagocytosed the infected erythrocyte relative to the positive control. Median antibody levels are presented as orange horizontal bars for each disease severity group. Total number of participants in the acute group with severe malarial anaemia, n = 33 (31 for Var19); impaired consciousness, n = 55 (52 for Var19); hyperlactataemia, n = 31 (26 for Var19). Opsonic antibody levels in the different disease severity groups were compared against four PfEMP1 types: A. E8B3X, binding ICAM-1. B. P6A1, binding CD36. C. R29, associated with rosetting. D. VAR19, binding EPCR. Sev, severe; BCS, Blantyre coma score; hyperlact, hyperlactataemia; Conv, convalescent.

5.4 Discussion

Different PfEMP1 variants encoded by different *var* gene groups have been associated with malaria. Group B and C *var* groups encode CD36-binding PfEMP1 [136]. Group C *var* genes were associated with asymptomatic malaria in PNG [148, 149], while group A PfEMP1 variants that do not bind to CD36 were associated with severe clinical disease, including cerebral malaria (impaired consciousness) [150, 154]. Expression of group B *var* genes (as well as group A and those *var* genes with a complex head structure) was shown to be associated with clinical malaria in PNG [148, 149]. Similarly, group B (and A) *var* gene transcripts were higher in severe (as well as clinical) malaria in Africa [151], while group B *var* genes were also associated with cerebral malaria (compared to mild or severe malarial anaemia cases) in Africa [152]. In chapter 4, we found antibodies to recombinant CIDRα1 domains binding EPCR to be acquired in older children with severe malaria in PNG. However, recombinant domains may expose epitopes that may not be normally observed in vivo in a full length PfEMP1 protein. This was demonstrated in a recent study, where interaction between IE and EPCR mediated by DC8 or DC13 containing PfEMP1 was different under physiological flow conditions and in recombinant proteins [126].

We measured opsonising antibody levels in the same PNG population as the one in chapter 4, to determine differences by disease severity in the opsonic antibody levels against whole PfEMP1 proteins expressed by parasite IE. These IE were selected for specific PfEMP1-receptor binding (in vitro), representing non-CD36 binding and CD36-binding PfEMP1 and include an ICAM-1 binder (which also binds CD36), CD36 binder, rosetting IE line and an EPCR binder. Although several *var* gene variants are expressed in early, ring stages of the parasite, only one *var* gene variant is predominantly expressed in later stages and is associated with a specific adhesion phenotype [118]. Opsonic antibody levels were compared between children with severe malaria and uncomplicated malaria at presentation and during convalescence and in age-matched healthy controls against the four PfEMP1 types.

We first divided the population into those having high antibody responses (% phagocytosis > median % phagocytosis) and low antibody responses (% phagocytosis ≤ median % phagocytosis) for each IE line tested. Proportion of children with high antibody responses (high antibody responders) against the four PfEMP1 types were then compared by disease severity. Next, we compared the opsonising antibody levels (that is % phagocytosis, determined based on percentage of THP-1 cells that have phagocytosed the IE relative to the positive controls) between children with severe malaria and

uncomplicated malaria at both timepoints and assessed change in antibody levels from presentation to convalescence for either form of malaria. Antibody levels in children with a particular severe syndrome were also compared to children having uncomplicated malaria. We also analysed differences in opsonic antibody levels in children within the severe malaria group having different severe malaria syndromes. These results have been discussed for each parasite IE line separately in the following paragraphs.

5.4.1 ICAM-1-binding PfEMP1-E8B3X parasite line

It is important to note that the results for antibodies (in severe malaria children) measured against the IE line E8B3X -selected for ICAM-1 binding- should be discussed with caution as this IE line also binds to CD36. Although the exact PfEMP1/ *var* type of the E8B3X IE line used in the current study is not known, based on previous qPCR data using E8B (selected for ICAM-1 binding) (done by Dr. Michael Duffy and group [326]) and the study by Lennartz and group [135] we think that this could be a group B ICAM-1 binder. *Var* transcription studies on this IE line are required to confirm this. ICAM-1-binding was shown to be facilitated by DBLβ domains belonging to group A or B/A, which contains a conserved sequence motif or belonging to groups B or C, which lacks the conserved motif [131, 135, 136]. Further, transcripts of ICAM-1-binding PfEMP1 (having the conserved sequence motif), either dually binding EPCR and belonging to DC13 [125] or belonging to DC4 [133] or belonging to other group A ICAM-1 binders not associated with any DC, were also shown to be upregulated in cerebral malaria [135]. The study from Avril and group [125] also showed that the ICAM-1 and CD36 dual binding IE line bound to resting brain endothelial cells to a lower extent compared to the EPCR and ICAM-1 dual binding parasite IE line. Taken together, the ICAM-1/CD36 IE line used in our present study may not be a representative of cerebral malaria causing IE line but could be a representative of uncomplicated malaria. Results from early studies looking at relations between ICAM-1 binding and disease severity have been varied. While previous studies from Africa have shown ICAM-1-binding PfEMP1 to be associated with cerebral malaria [156, 157], one study from Malawi showed an inverse relation between ICAM-1 binding and cerebral malaria [158]. Whether the antibody profile observed in our study is driven by the dual binding phenotype associated with E8B3X IE line (ICAM-1/CD36 vs ICAM-1-EPCR) would need to be further studied.

On comparing the proportion of high antibody responders against the ICAM-1 binding IE (Figure 5.2A), no differences were found between children with severe malaria or uncomplicated malaria at presentation or during convalescence. However, on comparing the actual opsonising antibody levels between the different study groups (Figure 5.3),

antibody levels were higher in children with severe malaria than uncomplicated malaria at presentation. Antibody levels declined (non-significantly) in severe malaria cases from presentation to convalescence. Together, these results suggest that antibodies observed in severe infection at acute time point could be a result of previous malaria infection, levels of which gradually declined over time due to lack of antibody boosting by current infection. As speculated in the previous paragraph, these results thus suggest that parasites expressing ICAM-1 (and CD36) binding PfEMP1 may not be responsible for the current severe infection and that these PfEMP1 types may not be associated with severe malaria in PNG children. This was further supported by our results from antibody levels in children with uncomplicated malaria. In children with uncomplicated malaria in our study, opsonic antibody levels to E8B3X were boosted from presentation to convalescence (Figure 5.3), suggesting children with uncomplicated malaria to be infected by parasites expressing ICAM-1 (and CD36) binding PfEMP1 and antibodies against them to function through phagocytosis.

Anti-DC4 antibodies inhibited the adhesion of PfEMP1 and ICAM-1 and were shown to be cross-reactive [133]. In PNG children with clinical malaria, higher levels of antibodies targeting the ICAM-1-binding PfEMP1 domain (DBLβ3 which is non-DC4 and occurs along with the EPCR binding CIDRα1 domain) resulted in reduced rates of clinical malaria as compared to children with lower levels of these antibodies [134]. Association of ICAM-1-binding PfEMP1 transcripts with malaria in PNG and antibody immunity studies for these PfEMP1 types are limited. Further, antibodies targeting ICAM-1-binding PfEMP1 have not been directly associated with protection from severe malaria. Thus, the current study is one of the first to determine associations between antibody immunity and ICAM-1 (CD36)-binding PfEMP1 by disease severity.

One limitation in using the E8B3X IE line in our study is that it does not exclusively bind to ICAM-1 but also binds to CD36. Although CD36 binding is lower than ICAM-1 binding (appendix IV), the presence of CD36 binding domain may influence the PfEMP1 epitopes exposed to antibodies. Measuring antibody responses against a parasite IE line expressing DC4 or DC13 ICAM-1 binders (which have previously been implicated in severe malaria) in cohorts such as the present one may help overcome this limitation and corroborate our current findings.

Thus, our results suggest uncomplicated malaria to be associated with antibody immunity against ICAM-1-binding PfEMP1 and antibodies to function through opsonic phagocytosis in PNG children. These results also suggest that parasites expressing ICAM-1-binding

PfEMP1 (similar to E8B3X which also binds CD36) may cause uncomplicated malaria but not severe malaria in PNG.

5.4.2 CD36-binding PfEMP1-P6A1 parasite line

Previous studies have linked CD36-binding IE with uncomplicated malaria [156-158]. In PNG, children with asymptomatic malaria were shown to have high transcript levels of group C *var* genes [148, 149]. In a different study, groups B and C encoding PfEMP1 were shown to bind CD36 [136]. Taken together, these studies have linked PfEMP1 binding to CD36 and linked expression of groups B and C *var* genes with uncomplicated malaria. In antibody immunity studies, seroreactivity to PfEMP1 that were CD36-binding and belonged to group B/C *var* groups was associated with uncomplicated malaria in Papua [161]. A study from Mali also found differences in seroreactivity against CD36-binding PfEMP1 fragments in children with cerebral malaria and uncomplicated malaria at presentation, suggesting their importance in protection from severe malaria, but requiring further investigation [214].

When we compared the proportion of children with high opsonic antibody responses against CD36-binding PfEMP1 (Figure 5.2B), those with severe malaria had a higher proportion of high antibody responders than those with uncomplicated malaria. Such differences were only observed at presentation and not during convalescence. On comparing the opsonising antibody levels between the different disease severity groups (Figure 5.4), the antibody levels declined from presentation to convalescence in cases of both severe malaria and uncomplicated malaria. Together, these results suggest that the opsonising antibodies observed at presentation could be a general repertoire of cross-reacting antibodies or antibodies due to previous exposure, levels of which declined with time due to lack of boosting by specific antigen types in the current infection and short half-life of antibodies [238]. One could also speculate that the *var* gene expression of some of the parasites causing both severe and uncomplicated malaria in these children could have been broadly similar to the P6A1 IE line tested.

Thus, CD36-binding PfEMP1 are recognised by broadly cross-reacting opsonising antibodies at the start of either severe or uncomplicated infection in PNG. These results are also in line with our results from chapter 4, where we did not observe antibodies acquired against CD36-binding CIDR domains to differ by disease severity in PNG children.

5.4.3 PfEMP1 associated with rosetting-R29 parasite line

Rosetting, that is binding of an IE with two or more uninfected erythrocytes, is encoded by group A *var* genes [149]. This phenotype was shown to be associated with severe malaria in several studies from Africa [153, 164-166]. Two studies, one from PNG and one from Malawi, found no associations between rosetting and severe malaria [158, 167].

When we compared the percent high antibody responders against the rosetting parasite IE line (figure 5.2C), the proportion of children with high antibody responses was similar in all the disease severity groups. On comparing levels of opsonic antibodies in children with severe malaria and uncomplicated malaria (figure 5.5), we found antibody levels to be higher in children convalescent from uncomplicated malaria compared to those with severe malaria. Such differences were not observed in acute plasma. Further, while we did not observe differences in opsonising antibody levels from presentation to convalescence for uncomplicated malaria, the antibody levels declined with time for severe malaria. These results suggest that presence of antibodies functioning through opsonic phagocytosis in uncomplicated malaria cases may be due to previous exposure to rosetting IE or they could be cross-reactive antibodies. But it is unlikely that the current severe infection was caused by rosetting parasites.

Our results are in line with a previous study demonstrating that antibodies against majority of the extracellular domains of PfEMP1 associated with rosetting could induce phagocytosis of the IE [271]. In a Kenyan study, children with severe malaria did not show associations between antibodies to IE and the rosetting phenotype [150].

The overall conflicting results in linking severe malaria with rosetting or anti-rosetting antibodies could be because of the interplay between the different VSAs on the IE (PfEMP1, RIFINS and STEVORS) in rosetting and host factors such as the ABO blood group [101]. In PNG, the association between rosetting and severe malaria is further confounded by lack of one of the major receptors required for rosetting: CR1, in a proportion of the population [327].

Thus, our results suggest that although parasites mediating rosetting of IE may not be the cause of the current severe malaria infection and the anti-rosetting, opsonising antibodies observed could be either cross-reactive or due to earlier exposure.

5.4.4 EPCR-binding PfEMP1- Var19 parasite line

PfEMP1s binding to EPCR through their CIDRα1 domains [63, 128] have been implicated in severe malaria in several studies. Transcripts of PfEMP1 or PfEMP1 containing

CIDRα1 domains were upregulated in severe malaria studies in children and adults. High expression levels of EPCR-binding PfEMP1 (DC8 or non-DC8) have been associated with severe malaria or severe malaria syndromes such as cerebral malaria and severe anaemia in children from Africa [120, 147, 159, 160] or PNG [161] or in severe malaria cases in adults from Papua [141] or India [138].

In the current study, when we compared the proportion of children with high opsonic antibody responses against the EPCR-binding (DC8) parasite IE line (figure 5.2D), we found children with high antibody responses were more common in the uncomplicated malaria group than the severe malaria group at presentation and during convalescence. Next, we compared the opsonic antibody levels against this IE line in all the disease severity groups (figure 5.6) and found children with uncomplicated malaria to have higher opsonising antibody levels than children with severe malaria at both time points. Our results indicate a protective role for antibodies targeting EPCR-binding PfEMP1, which function through phagocytosis. Thus, children with uncomplicated malaria may have been previously exposed to parasite strains binding EPCR and hence were protected from a severe infection in the current episode. The opsonic antibody levels increased from presentation to convalescence in our study in children with severe malaria but not in children with uncomplicated malaria. This suggests that EPCR-binding PfEMP1 could be the cause of the current severe infection as the antibody levels were boosted with time. These results are also in agreement with a recent study from Mali, where children with cerebral malaria or severe anaemia at presentation had lower antibody levels as compared to convalescence against PfEMP1s containing CIDRα1 domains and not binding CD36 [214].

Antibodies against PfEMP1 belonging to DC8 and binding EPCR were shown to be acquired early in life [120, 229] and capable of disrupting the binding of CIDRα1 to EPCR [128]. However, studies describing the role of opsonising antibodies targeting EPCR-binding PfEMP1 in protection from severe malaria are limited, with one recent study demonstrating that anti-PfEMP1 antibodies function partly through phagocytosis in these PNG children [323].

Thus, we found antibodies against EPCR-binding PfEMP1s to be higher in uncomplicated malaria than severe malaria in PNG children and to function through opsonic phagocytosis, suggesting these antibodies may protect from severe malaria in children from PNG. These results also suggest that parasites expressing EPCR-binding PfEMP1 domains may be responsible for the current severe infection in these children. These findings are in line with our results from chapter 4, where we found antibodies against the

EPCR-binding CIDRα1 domains of PfEMP1 were higher in children with severe malaria compared to uncomplicated malaria during convalescence. While our results from chapter 4 examined acquisition of antibodies to recombinant CIDR domains binding EPCR, the current study measured the quantities of functional antibodies to IE expressing the entire PfEMP1 molecule binding EPCR. Together, both results point to the role of EPCR-binding PfEMP1 in the pathogenesis of severe malaria and importance of antibody immunity against these PfEMP1s.

5.4.5 Antibody responses in community controls

Opsonic antibody levels in healthy, age-matched community controls were significantly lower than children with severe malaria or uncomplicated malaria at presentation and during convalescence for all four PfEMP1 types tested (figures 5.2, 5.3-5.6). Our results suggest that the healthy individuals have most probably not been exposed to malaria and do not have protective immunity either [214, 328]. Low antibody levels could also be attributed to absence of current malaria infection and lack of antibody boosting. Comparing antibody levels in healthy community controls with current infection (for example asymptomatic) to healthy individuals without a current infection could help us address this hypothesis.

5.4.6 Antibody responses to severe malaria syndromes

In young children with severe malaria from Africa, impaired consciousness, respiratory distress and severe anaemia were shown to be the commonly overlapping syndromes, with impaired consciousness and respiratory distress being the strongest predictors of mortality [46]. In our study, respiratory distress (or acidotic breathing) and hyperlactataemia (blood lactate > 5 mmol/L) were considered as surrogates for metabolic acidosis. We examined levels of opsonic antibody in children with severe anaemia, impaired consciousness (BCS 0-4) or hyperlactataemia compared to children with uncomplicated malaria.

Analyses for the ICAM-1-binding IE line (which also binds CD36) showed opsonic antibody levels to be higher in children with severe anaemia or impaired consciousness compared to children with uncomplicated malaria at presentation but not during convalescence (figures 5.7A and 5.8A, figures 5.9A and 5.10A). For children with hyperlactataemia, similar trend in opsonic antibody levels was observed, however the differences were not significant (figures 5.11A, 5.12A). Together, these observations could suggest that opsonising antibodies against ICAM-1/CD36-binding PfEMP1 are a result of previous malaria exposure and may not be associated with severe malaria

syndromes in the current infection. Previous studies have linked expression of ICAM-1-binding PfEMP1 (which also binds EPCR) with a higher risk of developing cerebral malaria [135]. As discussed in section 5.4.1, the IE line used in the current study - although selected to bind to ICAM-1, also binds CD36- could be a representative of uncomplicated malaria. Thus, we cannot link the antibody levels measured in the present study directly with the severe malaria syndromes.

For CD36-binding PfEMP1 type, opsonic antibody levels did not differ significantly between children with severe anaemia or impaired consciousness or hyperlactataemia and children with uncomplicated malaria (figures 5.7-5.12B). These results suggest a similar exposure to CD36-binding PfEMP1 for both severe and uncomplicated malaria.

In an early study, anti-rosetting antibodies were shown to be lacking in Gambian children with cerebral malaria, but present in those with uncomplicated malaria suggesting a protective role for these antibodies [226]. In the present study, opsonic antibody levels against the rosetting IE were higher in children with uncomplicated malaria compared to children with either severe anaemia or impaired consciousness or hyperlactataemia at presentation and during convalescence (figures 5.7-5.12C). However, the differences were significant only for plasma collected at convalescence and not at presentation. Considering that antibody levels declined from presentation to convalescence for children with severe malaria (figure 5.5), these results suggest that the opsonising antibodies observed during convalescence for the severe syndromes are cross-reactive or due to previous exposure.

We found the opsonic antibody levels against EPCR-binding PfEMP1 to be higher in children with uncomplicated malaria compared to children with severe anaemia or impaired consciousness or hyperlactataemia (figures 5.7-5.12D). Such differences were observed at presentation and convalescence, except for severe anaemia, where the difference was not significant at presentation. At presentation, there was a stronger evidence for differences in opsonising antibody levels between children with uncomplicated malaria and children with impaired consciousness or hyperlactataemia compared to evidence for differences in antibody levels between uncomplicated malaria and severe anaemia cases. This could suggest a stronger association of opsonic antibodies against EPCR-binding PfEMP1 with protection from impaired consciousness and hyperlactataemia compared to severe anaemia. While studies examining differences in antibody levels based on individual severe malaria syndromes are limited, transcription levels of EPCR-binding PfEMP1 domains were shown to be upregulated in severe

syndromes (including but not restricted to cerebral malaria or any other specific syndrome) in several studies such as: in children from Africa [147, 160] and PNG [161].

Thus, the patterns of antibody responses observed on comparing children with a particular severe syndrome and children with uncomplicated malaria are similar to the antibody patterns observed on comparing the severe malaria group (without stratifying by severe syndromes) and uncomplicated malaria group for the respective PfEMP1 types tested. Hence, we next compared antibody levels among the children with severe malaria to determine if the antibody levels differed by the type of syndrome presented. For all four PfEMP1 types tested, the opsonic antibody levels were similar across the three severe syndromes: severe anaemia, impaired consciousness and hyperlactataemia at presentation and during convalescence (tables 5.4 and 5.5, figures 5.13 and 5.14).

Thus, in our study we did not find antibody immunity to be specifically associated with a particular severe malaria syndrome or protection from the same. Naturally acquired immunity to severe malaria syndromes can be syndrome-specific or non-syndrome-specific [214].

Of the four PfEMP1 types tested, we found that antibodies against the EPCR-binding PfEMP1 could be associated with protection from severe malaria and severe malaria syndromes. We also found opsonic antibodies against severe malaria to be non-syndrome specific, and that having antibodies against PfEMP1 types causing severe malaria (inclusive of all syndrome types) may protect from individual severe malaria syndromes as well.

5.4.7 Summary of discussion and conclusion

Thus, this study helped us understand differences in levels of opsonising antibodies - against parasites expressing different dominant PfEMP1 types- by disease severity in children from PNG. We measured these antibodies against two PfEMP1 types belonging to group A or B/A (rosetting and EPCR binders, implicated in severe malaria) [63, 120, 138, 141, 147, 149, 153, 159, 160, 164-166], one belonging to either group A, B or C (ICAM-1-binders, of which the group As have been implicated in severe malaria) [131, 133, 135, 136] and one belonging to group B/C (CD36 binders, implicated in uncomplicated malaria) [129, 130, 136, 156-158]. We did not find any evidence for differences in antibody levels against the CD36-binding PfEMP1 type in cases of severe or uncomplicated malaria. We found weak evidence for opsonising antibodies against the ICAM-1 (and CD36)-binding PfEMP1 to be associated with uncomplicated malaria. We also found that opsonising antibodies observed during convalescence against the

rosetting IE may be due to previous exposure or cross-reactive antibodies. However, strongest evidence was observed for opsonising antibodies targeting the EPCR-binding PfEMP1. Antibodies against the EPCR-binding PfEMP1 were higher in uncomplicated malaria cases compared to severe malaria, thus suggesting a role for these antibodies in protection from severe malaria and its syndromes as well. Differences in antibody levels observed against the ICAM-1 (plus CD36) binders or rosetting IE could be attributed to previous exposure to these PfEMP1 types or cross-reactive antibodies. Although naturally acquired antibody immunity to different PfEMP1 domains was shown to vary between domains, antibodies targeting group A PfEMP1s could be cross-reactive due to similarities in their exposed epitopes [329]. This is also supported by a previous antibody agglutination study against PfEMP1, which showed convalescent sera to have both variant-specific as well as cross-reactive antibodies against PfEMP1 and attributed this cross-reactivity to conserved epitopes in PfEMP1 domains [206].

Therefore, with respect to the hypothesis and aims presented in chapter 2, we can infer that the antibody profile differed between the severity groups based on the type of PfEMP1 being targeted. Further, as hypothesised, the antibody levels increased from presentation to convalescence, which was more evident against PfEMP1 types which seem to be similar to the PfEMP1s causing the severe infections.

Limitations include small numbers of children with any one specific syndrome, thus restricting us in our analyses to understand antibody immunity to these syndromes. The parasite IE lines used for measuring antibody levels are only a representative of a particular PfEMP1 type dominantly expressed by the parasite. These IE also express other VSA families, viz. STEVORs and RIFINS. Although PfEMP1 on the IE was shown to be the major target of antibody immunity (and also the major target of opsonising antibodies in the current population [323]), role of other VSAs in eliciting antibody responses cannot be ignored [190]. The opsonic antibody levels measured in our study could be a cumulative effect of all VSA families on antibody immunity and cannot be attributed to PfEMP1 alone. Future studies determining other roles for antibodies targeting the EPCR-binding PfEMP1 such as inhibition of CIDRα1-EPCR binding as well as dissecting the role of other VSAs in this immunity are required.

Thus, we found antibodies targeting EPCR-binding PfEMP1 to function partly through phagocytosis with potential to protect from severe malaria in young children from PNG. Such data for opsonising antibodies have been lacking for children from Asia-Pacific regions. This study also provided insights on the type of PfEMP1 proteins expressed by parasites circulating in these regions and development of antibody immunity against the

proteins. Understanding anti-PfEMP1 immunity in these regions is essential for future vaccine studies as it may or may not be similar to antibody immunity in African regions.

6 Chapter 6: Expression of PfEMP1 domains using wheat germ cell-free protein synthesis system and evaluation of them as targets of antibody immunity in individuals with severe or uncomplicated malaria or community controls

6.1 Introduction

PfEMP1 is an important immune target for malarial immunity. PfEMP1 proteins are multi-domain, clonally variant and expressed on the surface of the IE, where they are exposed to host antibodies. They are encoded by ~60 *var* genes per parasite genome [90, 114, 115]. The protein is mainly composed of DBL and CIDR domains (Figure 1.4). The DBL domains can be further divided into six major classes, DBL α, β, γ, δ, ε and ζ; while the CIDR domians have five sub-classes: α, β, γ, δ and pam [110, 121] Domain cassettes (DC) of PfEMP1 have also been defined as a conserved arrangement of two or more PfEMP1 domains [110]. Different domains and domain sub-classes of PfEMP1 can bind to various receptors expressed by host cells. These have been summarised in table 1.2.

Var genes can be divided into three major groups, A, B and C. Several studies have investigated the association between the expression of these three major *var* types, A, B and C, and severe malaria. Group A and the chimeric group B/A PfEMP1 transcripts have been associated with severe malaria [150, 155]. Expression of group B *var* genes (as well as group A and those *var* genes with a complex head structure) was shown to be associated with clinical malaria in PNG [148, 149]. Similarly, group B (and A) *var* gene transcripts were higher in severe (as well as clinical) malaria in Africa [151], while group B *var* genes were also associated with cerebral malaria (compared to mild or severe malarial anaemia cases) in Africa [152]. Group C *var* genes were associated with asymptomatic malaria in PNG [148, 149]. Expression of conserved sets of PfEMP1 variants based on *var* gene groupings have been associated with high pathogenecity and low host immunity [154]. Antibody immunity against various PfEMP1 domains belonging to different *var* groups was shown to be acquired in an ordered fashion: first against the group A variants, followed by group B and C PfEMP1 types [220]. Studies of antibody immunity against the different PfEMP1 domains have been summarised in table 1.2.

PfEMP1 is a surface antigen and is implicated in the pathogenesis of severe malaria. Immunity to severe disease is acquired relatively rapidly, consistent with the existence of a restricted set of severe malaria associated, virulent antigens [217, 330, 331]. PfEMP1 is the primary antigen expressed on the infected erythrocyte surface that elicits immunity associated with protection, thus making PfEMP1 a potential vaccine candidate [190].

Antigenic variation of PfEMP1 [114], determining whether there are any sufficiently conserved, protective PfEMP1 antigens, limited knowledge of PfEMP1s tertiary and quarternary structure [170] and difficulty in expressing correctly folded, non-glycosylated, soluble, recombinant PfEMP1 [314] are some of the problems currently impeding development of PfEMP1 as a vaccine candidate. The structures of certain PfEMP1 domains (sometimes in relation to their binding receptors) have been previously studied using different approaches [128, 135, 153, 207].

Due to difficulties in expressing the full length PfEMP1, very few studies have studied associations between immune responses to the entire PfEMP1 protein and severe malaria. Hence, most of the *var* transcript and antibody immunity studies have relied on identifying associations between specific PfEMP1 domains or domain cassettes in susceptibility to, or protection from, severe malaria [141].

In chapter 4, we measured antibody responses in PNG children against 35 recombinant CIDR domains of PfEMP1. We found antibodies against EPCR-binding CIDR domains to be significantly higher in older children who had recovered from severe malaria as compared to children who had recovered from uncomplicated malaria. In chapter 5, we found that opsonising antibody levels against erythrocytes infected with parasites expressing EPCR-binding PfEMP1s were significantly boosted from presentation to convalescence following severe malaria infection. Strong evidence for such boosting was not observed against other PfEMP1 types.

Based on these results, we next wanted to assess antibody responses against other domains and domain sub-classes of PfEMP1 and determine if antibody levels differ based on disease severity. We wanted to examine associations between antibody responses and PfEMP1 domains newly identified as being implicated in severe malaria (as described later in section 6.2.3). We also wanted to look for parallels in antibody responses to PfEMP1 in malaria infected adults and children from other geographical regions and our results from chapter 4 and 5.

Previous *var* transcript studies depended on *var* sequences derived from laboratory parasite lines for primer design and PCR amplification. While these studies increased our knowledge of the role of these domains in severe disease, a better understanding of these domains in a clinical setting is required. In the current study, expressed *var* sequences from clinical isolates (parasites infecting Papuan individuals with either severe or uncomplicated malaria) were assembled using RNAseq (irrespective of the *var* gene homology in the sequenced laboratory isolates), and analysed using novel bioinformatics analyses. This in turn led to identification of *var* gene sequences which were differentially

expressed in severe or uncomplicated malaria at the multi-domain, single-domain or sub-domain level [141].

We cloned 34 such PfEMP1 domains in total (upregulated in severe or uncomplicated malaria clinical isolates), which were then expressed by our collaborators, Professor Takafumi Tsuboi and Dr. Eizo Takashima, using the wheat germ cell free protein synthesis systems (WGCFS). 32 PfEMP1 domains were successfully expressed. We used a multiplex assay using MAGPIX to measure antibody responses to these 32 domains in two separate study populations: Papuan individuals with severe or uncomplicated malaria at presentation; and Malawian children with cerebral or uncomplicated malaria at presentation and follow-up.

6.2 Methods

6.2.1 Study population

We measured antibody levels against recombinant PfEMP1 domains of interest in study populations from two geographical locations for this chapter: Timika, Papua, Indonesia, and Blantyre, Malawi.

Indonesia

Study participants recruited for this study were individuals aged 4-61 years presenting with either severe or uncomplicated malaria at a healthcare facility in Timika, Papua Province, Indonesia. This population has been previously described in [141, 161] and has been summarised in table 6.1 and 6.2. Peripheral parasitaemia in addition to one or more modified WHO criterion of severe disease as described in [332] was used to define severe malaria in these individuals. 25 patients with severe malaria presented with parasitaemias > 1000/µl, two patients with severe malaria had parasitaemias < 1000/µl and parasite density data for one patient with severe malaria was not available. These parasite density levels were previously described as thresholds that could predict clinical disease in north Papua [333] and thus would include individuals with severe malaria and uncomplicated malaria (but reduce the possibility of incidental parasitaemia). Venous blood samples were collected from each participant and plasma (for antibody studies) and red blood cells (for *var* gene analysis) were isolated from each sample [141, 161]. The study population used for antibody measurements is the same as the population used to determine differential *var* gene expression by [141] (we had more number of plasma samples for antibody measurements than were used for *var* transcription). Of note: a Glasgow coma score of < 11 was used for cerebral malaria assessment in the

Papuan study [332], while for the Malawian children a BCS score ≤ 2 was used (as described below).

Plasma samples at presentation from 28 individuals with severe malaria and 35 individuals with uncomplicated malaria were used to measure antibody levels against 22 PfEMP1 domains upregulated in severe malaria (SM-derived domains), 10 domains upregulated in uncomplicated malaria (UM-derived domains), 2 *P. falciparum* control proteins and a tetanus toxin.

Malawi

Study participants were recruited as a part of a case-control study conducted in Blantyre, Malawi (described in [334]). Children aged 5-148 months presenting with either cerebral malaria or uncomplicated malaria at Queen Elizabeth Central Hospital in Blantyre were included in this study. Microscopy along with RDT and fever was used to determine malaria cases. Children with cerebral malaria were assigned a BCS score of 0, 1 or 2 based on the verbal, motor and gaze responses to stimuli in these children [335]. Children with uncomplicated malaria had normal consciousness or a BCS of 5 and were treated as outpatients. Parasitaemia was also used to categorise the kids as cerebral or uncomplicated malaria (table 6.3). Children with HIV or malnourishment were excluded from the study. Venous blood was collected from these children at hospital presentation (acute) and 4 weeks later (follow-up). Venous blood from age-matched, healthy children (community controls) was collected at the Ndirande Health Centre, Blantyre. These children were malaria negative by RDT and clinically well. Plasma was separated from the blood samples by centrifugation and stored at -80^{o}C until shipping to Melbourne.

Thus, samples were categorised into 5 groups based on disease severity. 119 children with cerebral acute malaria and 68 children from the follow-up group, 118 children with uncomplicated acute malaria and 65 children in the follow-up group along with 101 children in the community controls group were tested for antibody levels against the various PfEMP1 domains. These domains included 22 domains upregulated in severe malaria, 10 domains upregulated in uncomplicated malaria and 3 control proteins.

6.2.2 Ethics

For the study population from Papua, Indonesia, written, informed consent was obtained from each participant. Ethical approval was obtained from Eijkman Institute Research Ethics Commission (project number 46) from Indonesia, Melbourne Health Human Research Ethics committee (project number 2010.284) and Human Research Ethics

Committee of the NT Department of Health & Families and Menzies School of Health Research, Darwin (HREC 2010-1396) in Australia [141].

Ethical approval for the Malawian study population was obtained from the College of Medicine Research and Ethics Committee (COMREC). Written, informed consent was obtained from parents/guardians of all participants included in the study. Melbourne Health Human Research Ethics Committee (HREC 2013.290).

6.2.3 Identifying PfEMP1 domains of interest

Identification of PfEMP1 domains upregulated in severe or uncomplicated malaria was done by Dr. Michael Duffy and his group. The RNAseq and bioinformatics analyses carried out have been described in detail in [141] and have been described briefly in this section. RNA was extracted using the RNeasy mini kit (Qiagen) from 23 patients with severe malaria and 21 patients with uncomplicated malaria from Timika, Papua, Indonesia. RNA libraries were synthesized using the NEBnext ultra directional RNA library prep kit for Illumina (New England Biolabs) and 125 bp end sequenced using RapidRun chemistry (Illumina). Various sequence and expression analyses were then carried out on these 44 transcriptome libraries. De novo assembly of *var* genes was carried out using the SoapDeNovo-Trans and Cap3 pipeline. *Var* gene differential expression analysis was carried out at the *var* transcript level*, var* type/domain level and *var* segment/homology block level. 4449 individual *var* transcripts were assembled from the patients' separate transcriptomes. These could be grouped by homology and expression profile into 82 clusters of related *var* transcripts. 5 of these clusters were upregulated in severe malaria. Hidden Markov Models [110] were used to identify *var* domain subtypes in the transcripts and read counts mapping to the domains were used to identify 16 domains that were upregulated in severe malaria. These domains were defined by bootstrapped phylogenetic trees and thus often were clustered at low sequence identity. To identify more conserved domain sequences that were upregulated in severe malaria the sequences were clustered at different sequence identity levels, and 15 clusters of domain subtypes were identified as upregulated in severe malaria. The same approach was used to identify 10 smaller homology block sequences that were upregulated in severe malaria.

6.2.4 Selection of patient isolates expressing PfEMP1 domains of interest and primer design

Representatives of the clustered domains subtypes that were upregulated in severe malaria (adjusted p-value <0.05 and log fold change ≥ 2) were amplified from the cognate patient genomic DNA (gDNA) (Appendix I, target/transcript column, S10 data from [141]).

Primers of length 15-33 nucleotides with a melting temperature (Tm) of 51-60^{o}C were designed using Geneious R10 (Biomatters, New Zealand) software. The primers were designed using flanking sequences at the start and end of our domains of interest. Restriction enzyme sites for XhoI (or BamHI) in the forward primer and XmaI (or NotI) in the reverse primer were incorporated. A stop codon was also incorporated in the reverse primer (before the restriction digestion site) to facilitate the termination of protein translation during expression. Precautions were also taken to maintain an even number of cysteine residues (wherever possible) in the protein sequence to facilitate proper folding of the translated protein.

Sequences for the two *P. falciparum* control proteins, AMA-1 and CSP, were obtained from Plasmodb and gDNA from one of the patients with severe malaria was used for amplification and subsequent cloning and expression. These proteins allowed us to control for the WGCFS method of protein production as well as to have non-PfEMP1 protein controls.

6.2.5 Experimental methods

The experimental procedures used for this chapter have been described in detail in chapter 3.

6.2.5.1 Cloning

Detailed methodology for cloning PfEMP1 domains of interest including the PCR amplification of the domains, purification and restriction digestion of the DNA, ligation and transformation of the domains into the expression vector, purification of recombinant plasmids and confirmation of successful transformation by DNA sequencing have been described in detail in section 3.6 of chapter 3. The primers used for PCR amplification and sequencing have been listed in appendices I and II.

6.2.5.2 Expression of PfEMP1 domains of interest

The WGCFS was used for the production of PfEMP1 domains of interest (including two control proteins by Professor Takafumi Tsuboi and Dr. Eizo Takashima (Ehime

University, Matsuyama, Japan) and CFS Co., Ltd, Matsuyama, Japan as previously described [316, 317], and has been briefly described in section 3.7 of chapter 3.

6.2.5.3 MAGPIX multiplex assay

6.2.5.3.1 Carbodiimide coupling of protein to Bio-Plex magnetic carboxylated microspheres

The expressed PfEMP1 domains were coupled to Bio-Plex magnetic carboxylated microspheres/beads (Bio-Rad, 1.25 X 107 beads/ml) using carbodiimide coupling. The details of the coupling procedure have been described in section 3.8.1.1.1.

6.2.5.3.2 Antibody measurement using MAGPIX multiplex assay

Total IgG levels against 32 PfEMP1 domains of interest coupled to beads were measured in patient plasma from Malawian children and adults from Papua, Indonesia using the MAGPIX multiplex assay. Antibody levels against 2 *P. falciparum* control proteins: AMA-1 and CSP and commercially available tetanus toxin (also coupled to the beads) were also measured. The experimental procedure has been described in detail in section 3.8.1.1.2 of the materials and methods chapter.

6.2.6 Statistical analyses

Antibodies measured using this assay are expressed as median fluorescence intensities (MFI). Various non-parametric tests were used for analyses as the data are not normally distributed. Corrections for the multiple comparisons carried out in this chapter were not made. Hypotheses testing and clustering analyses using MATLAB were carried out by Dr. Amy Chung and Mr. Timon Damelang.

Statistical analyses were carried out using GraphPad PRISM5 software (La Jolla, CA, USA) and MATLAB version 2017b with the Statistics and Machine Learning Toolbox (MathWorks, Massachusetts, USA).

Machine learning models using MATLAB were used to determine antibody patterns or relationships between the antibody levels measured against the 32 PfEMP1 domains and 2 *P. falciparum* control proteins in both the study populations. This was followed by analysing the antibody responses in the plasma samples tested against the individual domains using a Wilcoxon-ranksum test, which is representative of a ‘traditional’ type of analysis, which complements the machine learning analyses (multivariate) and helps confirm the findings as either statistically significant or not.

Analyses of Papuan population

A supervised approach was used to analyse the antibody responses in the Papuan individuals presenting with either severe or uncomplicated malaria. Supervised analysis is an approach where the relationships between the features (antibody levels) is assessed in combination with known clinical outcomes [336]. Thus, signatures of measured features (antibody responses to different PfEMP1 domains) were associated with different clinical outcomes (example, severe and uncomplicated malaria). Elastic net (a feature selection analysis), was used to identify antibody signatures that best differentiate the different clinical outcomes. The optimum value of the tuning parameter "Alpha" was chosen as 0.4, such that the resulting model had the lowest minimum standard error (MSE) for prediction using 5-fold cross validation during model fitting. Partial least squares discriminant analysis (PLSDA), a type of supervised clustering analysis [337, 338], was applied to assess the differences between the antibody features identified by elastic net, between clinical groups. Prior to PLSDA analysis, data was normalized by mean centering and variance scaling. Leave one out cross validation (LOOCV) was performed during model assessment to test model predictions. These models helped us narrow down our large datasets and identify antibody responses to PfEMP1 domains to be further investigated as important targets of immune responses associated with different disease outcomes. To identify differences in antibody levels against all the PfEMP1 domains tested (n=32), antibody levels were also compared between individuals with severe malaria and uncomplicated malaria using Wilcoxon Rank Sum test ('traditional analysis').

Analyses of Malawian population

To analyse how antibody levels varied between Malawian children with cerebral malaria and uncomplicated malaria at two time points (hospital presentation/acute and follow-up), an unsupervised model using principal component analysis (PCA) was used. PCA allows us to determine signatures of antibody responses that result in most variation between our four sample groups without any information about clinical outcomes [336, 339]. A supervised PLSDA model (without any feature selection) was then used to identify associations between antibody responses and various PfEMP1 domains in 1) children with cerebral malaria and uncomplicated malaria at acute time point and 2) children with uncomplicated malaria at acute and follow-up time points. Because major associations/ clustering of antibody responses in the different disease severity groups were not observed with the PCA and PLSDA analysis (without feature selection), and due to time constraints, further analysis with feature selection (as done for the Papuan population)

was not carried out for this population. Wilcoxon Rank Sum tests were also used to compare antibody levels between children with cerebral malaria and children with uncomplicated malaria at each time point. The test was also used to compare change in antibody levels from presentation to follow-up in the two disease severity groups.

6.3 Results

6.3.1 Summary of study population

Papua, Indonesia

Total IgG levels were measured in plasma samples from 28 individuals with severe malaria and 35 individuals with uncomplicated malaria at presentation against a total of 32 PfEMP1 domains and 3 control proteins coupled to beads. Participants with severe malaria had a median age of 29 years, with 39.3% being females. Individuals with uncomplicated malaria had a median age of 22.5 years, with 45.7% being females. The Papuan study population has been summarised in table 6.1. Most of the patients with severe malaria presented with a single severe malaria syndrome such as cerebral malaria, jaundice, acute renal failure, hyperparasitaemia, or prostration, while a few individuals presented with two severe malaria syndromes. Numbers of individuals with each of these presentations have been summarised in table 6.2.

Blantyre, Malawi

Antibody levels were measured and analysed in plasma from 119 children with cerebral malaria at presentation and 68 children in the follow-up period. Similar measurements were made in plasma samples from 118 children with uncomplicated malaria and 85 children in the follow-up period. Plasma samples from healthy, malaria negative children (community controls) were also tested for total IgG levels. Antibody levels were measured against 22 PfEMP1 domains upregulated in severe malaria, 10 domains upregulated in uncomplicated malaria, 2 P. falciparum control proteins and 1 tetanus toxin. All available samples were measured.

At presentation with the disease, children with cerebral malaria had a median age of 48.5 months, with 44.5% being females; while children with uncomplicated malaria had a median age of 48 months, 41.5% being females. Children with cerebral malaria had a BCS score ranging from 0-2. Summary of the Malawian study population has been presented in table 6.3.

Table 6.1 Summary of Papuan study population categorized by disease severity.

Characteristic		Severe malaria (n=28)	Uncomplicated malaria (n=35)
Age (years)[a]		29 [19.5-34]	22.5 [18-25][b]
Gender[c]	**Female**	39.3 (11)	47.1 (16)[d]
	Male	60.7 (17)	52.9 (18)[d]
Haemoglobin (g/dL)[a]		11.7 [10.1-13.7]	12.3 [10.6-14.1][e]
Parasite density (parasites/µl)[a]		39400 [8600-252597][f]	27360 [16600-52800][g]

[a] data for age, haemoglobin (g/dL), and parasite density (parasites/µl presented as median [interquartile range].

[b] data available for n=34.

[c] data for gender presented as percentage (total number).

[d] data available for n=34.

[e] data available for n=34.

[f] data available for n=27.

[g] data available for n= 28.

Table 6.2 Summary of severe malaria syndromes in Papuan individuals with severe malaria.

Severe malaria syndrome	Severe malaria (n=28)
Cerebral malaria	17.9 (5)
Jaundice	21.4 (6)
Acute renal failure	3.6 (1)
Hyperparasitaemia	28.6 (8)
Prostration	10.7 (3)
Acute renal failure and ARDS	3.6 (1)
Jaundice and hyperparasitaemia	3.6 (1)
Jaundice and acute renal failure	7.1 (2)
Hyperparasitaemia and prostration	3.6 (1)

Data presented as percentage (total number)

ARDS, acute respiratory distress syndrome

Table 6.3 Summary of Malawian study population categorized by disease severity.

Characteristic	Cerebral malaria		Uncomplicated malaria		Community controls
	Acute (n=119)	**Follow-up (n=68)**	**Acute (n=118)**	**Follow-up (n=85)**	**(n= 101)**
Age (months)[a,b]	51 [34-77][c]	55 [37-82][d]	48 [25-85][e]	49 [26.8-85.5][f]	24 [12-67.5][g]
Gender[b,h] : Female	44.5 (53)	47.1 (32)	41.5 (49)	37.6 (32)	57.6 (57)[i]
Male	55.5 (66)	52.9 (36)	58.5 (69)	61.2 (52)	42.4 (42)[i]
Haemoglobin (g/dL)[a]	7.9 [7-9.2][j]	11.1 [9.7-11.9][k]	9.9 [8.7-11.4][l]	11.3 [10.4-12.1][m]	11.9 [11.1-12.8][n]
Parasite density (parasites/µl)[a,o]	4779 [414-93380]				

[a]Data for age, haemoglobin (g/dL), and parasite density (parasites/µl presented as median [interquartile range].

[b]Data for age and gender during follow-up as recorded during acute timepoint.

[c]age (months) data for cerebral acute group, n=109.

[d]age (months) data for cerebral follow-up group, n = 65.

[e]age (months) data for uncomplicated acute group, n = 117.

[f]age (months) data for uncomplicated follow-up group, n = 84.

[g]age (months) data for community controls group, n = 96.

[h]Data for gender presented as percentage (total number).

[i]Data for gender in community controls group, n=99.

[j]Data for haemoglobin in the cerebral acute group, n=114.

[k]Data for haemoglobin in the cerebral follow-up group, n=28.

[l]Data for haemoglobin in the uncomplicated acute group, n=115.

[m]Data for haemoglobin in the uncomplicated follow-up group, n=81.

[n]Data for haemoglobin in the community controls group, n=91.

[o]Parasite density data for cerebral follow-up, uncomplicated acute and follow-up and community controls not available. WHO thick blood film grading scale was used to assess the parasitaemia in the uncomplicated group while a ‘negative’ parasitaemia was used for the community controls.

6.3.2 PfEMP1 domains cloned and sequenced

28 PfEMP1 domains upregulated in severe malaria were selected to be cloned and expressed and tested as targets of antibody immunity. 16 domains downregulated in severe malaria (or upregulated in uncomplicated malaria) and 2 *P. falciparum* control proteins (AMA-1 and CSP) were also selected for expression. We were able to successfully clone 23 out of 28 PfEMP1 domains upregulated in severe malaria (SM domains) and 11 out of 16 domains upregulated in uncomplicated malaria (UM domains). Unsuccessful cloning of 5 SM domains and 5 UM domains could be attributed to primers not binding and thus unsuccessful PCR amplification or mutations introduced during cloning. The list of domains cloned and reasons for unsuccessful cloning have been represented in table 6.4. The domains cloned (and expressed) which have been presented in the following tables, have been assigned a DC in some cases. It is important to note that some of these domains also occur outside of these DCs or may not be part of any DC. See [141] for more details on the *var* transcription analyses for these domains and assignment of DCs.

Table 6.4 List of PfEMP1 domains of interest selected for cloning and expression and status of cloning.

Sequence name[a]	Domain name	Domain cassette[b]	*Var* group[c]	Cloning status	Remarks
SM1	CIDRα2.4.s.1		B	Cloned	
SM2	CIDRβ1.s.1	Non-DC4	B	Cloned	
SM3	DBLβ12.s.1	DC8	B/A	Cloned	
SM4	DBLβ3.s.1	Non-DC4	A	Cloned	
SM5	DBLβ3.s.2	DC4 or non DC4	A	Cloned	
SM6	DBLδ1.s.1		B	Cloned	
SM7	DBLδ1.s.2		B	Not cloned	PCR amplification from patient gDNA did not work.
SM8	DBLδ1.s.3		B	Cloned	
SM9	DBLδ1.s.4	DC8	B	Cloned	
SM10	DBLε2.s.1		B	Not cloned	PCR amplification from patient gDNA did not work.
SM11.1	DBLε3.s.1	DC11	B	Cloned	
SM12	DBLε9.s.1		B	Cloned	
SM13	DBLεpam5.s.1	VAR2	E	Cloned	
SM14	DBLγ3.s.1	DC9	B	Cloned	
SM15	DBLζ4.s.1	DC9	B	Cloned	
SM16	DBLα1.2		A	Not cloned	Cloned sequence did not match patient sequence.
SM17	DBLα1.5		A	Cloned	
SM18	CIDRα1.1	DC8	B/A	Cloned	
SM19	CIDRα1.6	DC4	A	Cloned	

SM20	DBLγ11		B/A	Cloned	
SM21	DBLα2	DC8	B/A	Not cloned	Mutations in the cloned sequence.
SM22	DBLε5	VAR1	A	Cloned	
SM23	CIDRγ9		B	Not cloned	Mutations in the cloned sequence.
SM24	DBLζ3		B/A	Cloned	
SM25	DBLβ13		B	Cloned	
SM26	CIDRγ12		B	Cloned	
SM27	DBLδ7		A	Cloned	
SM28	CIDRα2.6-DBLβ5		B	Cloned	
UM1	DBLα0.13.ns.1		B	Cloned	
UM2	DBLδ1.ns.2		B	Cloned	
UM3	CIDRα2.5.ns.1		B	Not cloned	Deletion mutation in the cloned sequence
UM4	DBLγ13.ns.1		B/A	Not cloned	Cloned colony did not have our domain of interest.
UM6	DBLα0.11.ns.1		B	Cloned	
UM8	DBLγ9.ns.1		B/A	Cloned	
UM9	CIDRβ1.ns.1		B	Not cloned	Cloned colony did not have our domain of interest.
UM14	DBLδ1.ns.6		B	Cloned	
UM18	DBLα0.3.ns.1		B	Cloned	
UM19	DBLδ1.ns.3		B	Cloned	
UM20	CIDRα3.1.ns.1		B/C	Cloned	
UM21	DBLα0.9.ns.1		B	Cloned	
UM33	DBLδ1.ns.5		B	Not cloned	Mutations in the cloned sequence

UM43	DBLpam2.ns.1	E	Cloned	
UM45	CIDRα1.7.ns.1	A	Cloned	
UM52	DBLα0.11.ns.2	B	Not cloned	Deletion mutation in the cloned sequence

[a]Total number of PfEMP1 domains cloned= 44 (excluding the 2 *P. falciparum* control proteins: AMA-1 and CSP).

[b]domain cassettes not known have been left blank.

[c]*var* groups as referenced in [110].

PfEMP1, Plasmodium falciparum erythrocyte membrane protein 1; SM, severe malaria; CIDR, cysteine-rich inter-domain region; DC, domain cassette; DBL, duffy binding-like; UM, uncomplicated malaria.

6.3.3 PfEMP1 domains expressed

23 PfEMP1 domains upregulated in severe malaria and 11 domains upregulated in uncomplicated malaria were shipped to Japan for expression using the WGCFS. The expression was done by Dr. Eizo Takashima and Professor Takafumi Tsuboi from Ehime University, Matsuyama, Japan. They were able to successfully express 22 PfEMP1 domains upregulated in severe malaria, 10 domains upregulated in uncomplicated malaria and the two *P. falciparum* control proteins AMA-1 and CSP. A list of proteins successfully expressed is in table 6.5 and SDS (sodium dodecyl sulphate) gels for the proteins expressed are in appendix III. SM20 and UM18 domains were not successfully expressed (Appendix III H and N). Thus, 22 SM domains, 10 UM domains, 2 *P. falciparum* control proteins and one commercially available protein, tetanus toxin, were used as targets to measure antibody levels in malaria exposed individuals from Malawi and Papua, Indonesia.

Quality control of the proteins was assessed by the SDS images (Appendix III). The ladder was used an indication of the approximate size of the proteins. The multiple bands observed in the SDS gel images are thought to be the truncated forms of the expressed proteins produced during affinity purification due to natural translational arrest of the N-terminal region of the protein (for example, appendix III E (SM15)). Further protein validation studies will be required to confirm the present findings using these proteins.

Table 6.5 List of PfEMP1 domains and control proteins expressed by WGCFS.

Sequence name[a]	Domain name	Domain cassette	*Var* group	Size-bp	Size-kDa	Concentration E1 (µg/µl)
SM1	CIDRα2.4.s.1		B	776	28.38	0.88
SM2	CIDRβ1.s.1	Non-DC4	B	756	27.72	0.43
SM3	DBLβ12.s.1	DC8	B/A	1422	52.14	0.34
SM4	DBLβ3.s.1	Non-DC4	A	1461	53.57	0.70
SM5	DBLβ3.s.2	DC4 or non-DC4	A	614	22.44	0.22
SM6	DBLδ1.s.1		B	1497	54.89	0.73
SM8	DBLδ1.s.3		B	1290	47.3	0.71
SM9	DBLδ1.s.4	DC8	B	1536	56.32	0.63
SM11.1	DBLε3.s.1	DC11	B	919	33.66	0.50
SM12	DBLε9.s.1		B	975	35.75	0.77
SM13	DBLεpam5.s.1	VAR2	E	942	34.54	0.17
SM14	DBLγ3.s.1	DC9	B	1126	41.25	0.74
SM15	DBLζ4.s.1	DC9	B	1316	48.18	0.81
SM17	DBLα1.5		A	1206	44.22	0.45
SM18	CIDRα1.1	DC8	B/A	774	28.38	0.46
SM19	CIDRα1.6	DC4	A	774	28.38	0.44
SM22	DBLε5	VAR1	A	1026	37.62	1.20

SM24	DBLζ3	B/A	1299	47.63	0.34
SM25	DBLβ13	B	1359	49.83	0.69
SM26	CIDRγ12	B	786	28.82	0.59
SM27	DBLδ7	A	1185	43.45	0.23
SM28	CIDRα2.6-DBLβ5	B	1398	51.26	0.33
UM1	DBLα0.13.ns.1	B	1308	47.96	0.18
UM2	DBLδ1.ns.2	B	1452	53.24	0.72
UM6	DBLα0.11.ns.1	B	1251	45.87	0.09
UM8	DBLγ9.ns.1	B/A	1155	42.35	0.70
UM14	DBLδ1.ns.6	B	1512	55.44	0.72
UM19	DBLδ1.ns.3	B	1545	56.65	0.48
UM20	CIDRα3.1.ns.1	B/C	885	32.45	0.81
UM21	DBLα0.9.ns.1	B	1251	45.87	0.06
UM43	DBLpam2.ns.1	E	1332	48.84	0.25
UM45	CIDRα1.7.ns.1	A	786	28.16	0.91
AMA-1	Pf control protein		1866	68.42	0.28
CSP	Pf control protein		1191	43.67	0.14

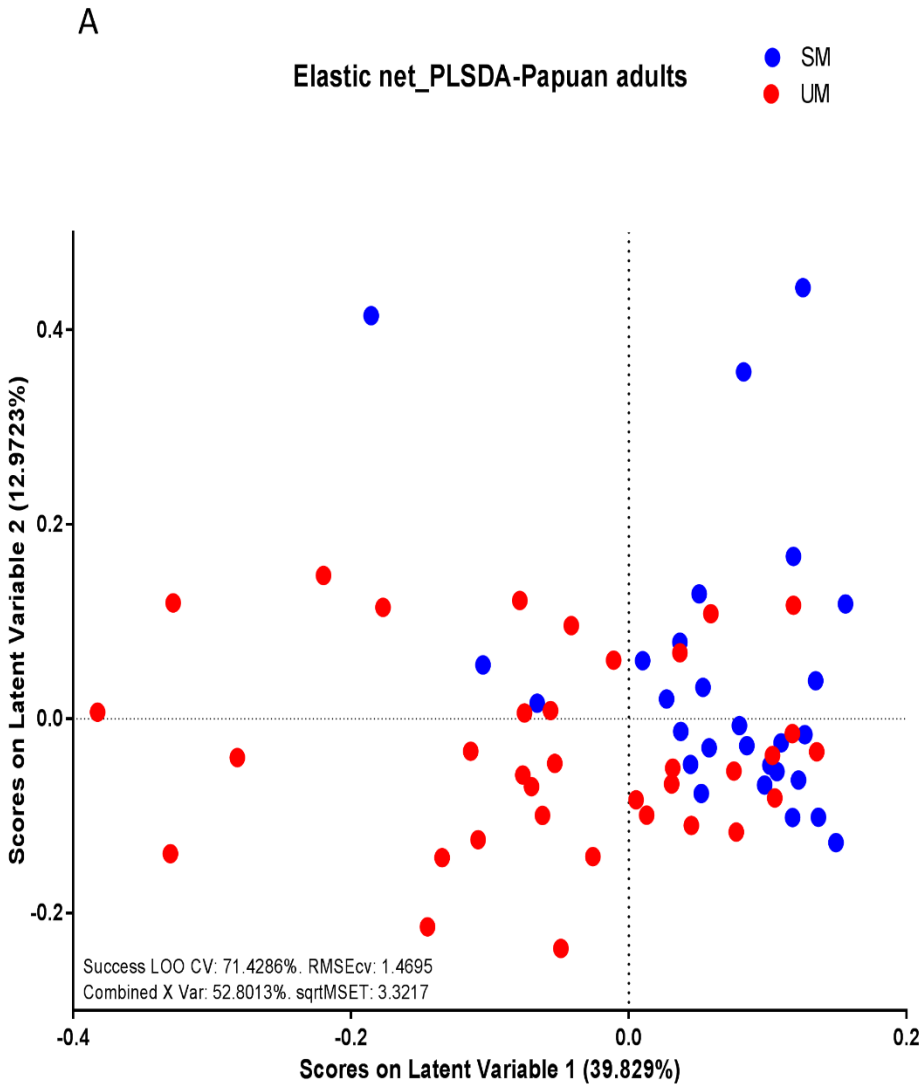
[a] SM 20 (size-kDa= 40.15) and UM18 (size-kDa= 44.11) were not expressed successfully and are not included in this list.

PfEMP1, Plasmodium falciparum erythrocyte membrane protein 1; WGCFS, wheat germ cell free synthesis systems; bp, base pairs; kDa, kilo daltons; E1, elution 1; SM, severe malaria; DC, domain cassette; UM, uncomplicated malaria; Pf, *P. falciparum*

6.3.4 Identifying relationships between antibody responses to PfEMP1 domains in Papuan individuals with severe and uncomplicated malaria

We wanted to assess associations between the antibody responses to 22 PfEMP1 domains upregulated in severe malaria and 10 domains upregulated in uncomplicated malaria in individuals from Papua, Indonesia having either severe or uncomplicated malaria. An elastic net, feature selection, followed by PLSDA model was used to determine these associations. This type of analysis was carried out by Dr. Amy Chung and Mr. Timon Damelang. This type of analysis helped us identify 7 features (PfEMP1 domains) that contributed the most to variation in antibody responses by severity in our dataset.

The results are presented in figure 6.1. Each dot represents the multivariate analysis of all antibody values generated against all 32 PfEMP1 domains (and 2 control proteins) for each individual belonging to a particular disease severity group (blue dots = severe malaria, red dots= uncomplicated malaria) across two dimensions. X-axis represents the latent variable score for the model in the first dimension and Y-axis represents the latent variable score along the second dimension of analysis. Antibody levels in individuals with severe malaria cluster somewhat separately from those with uncomplicated malaria (LOOCV = 71.4%, combined variance = 52.8%) (Figure 6.1A). Separate clustering is indicated particularly by red dots that are not overlapping with blue dots. The combined variance represents the variance along the X-axis and Y-axis (first and second dimension analysis). Most of the separation seems to occur along the first dimension (X-axis, variance = 38.8%), with clustering along the second dimension (Y-axis, variance = 12.9%) being much lower. Based on the position of the antibody features along X and Y axes (Figure 6.1B) in relation to clustering observed in Figure 6.1A, antibody responses in individuals with uncomplicated malaria were most prominently associated with one domain upregulated in uncomplicated malaria, UM2-DBLδ1 and six PfEMP1 domains upregulated in severe malaria: SM19-CIDRα1.6, SM17-DBLα1.5, SM15-DBLζ4, SM4-DBLβ3, SM1-CIDRα2.4 and SM18-CIDRα1.1 (that is, these domains, (in particular those domains whose transcripts were upregulated in SM) were associated with maximum variation in antibody responses by disease severity: ‘strong targets of protective immunity’) (Figure 6.1B). Based on the two axes, SM18-CIDRα1.1 domain seems be a representative of antibody responses in a few individuals with severe malaria (blue dots) out of the entire population, with majority of the other individuals with severe malaria clustering quite far from it.


A
Elastic net_PLSDA-Papuan adults
SM
UM
Scores on Latent Variable 2 (12.9723%)
Scores on Latent Variable 1 (39.829%)
Success LOO CV: 71.4286%. RMSEcv: 1.4695
Combined X Var: 52.8013%. sqrtMSET: 3.3217
0.4
0.2
0.0
-0.2
-0.4
-0.2
0.0
0.2

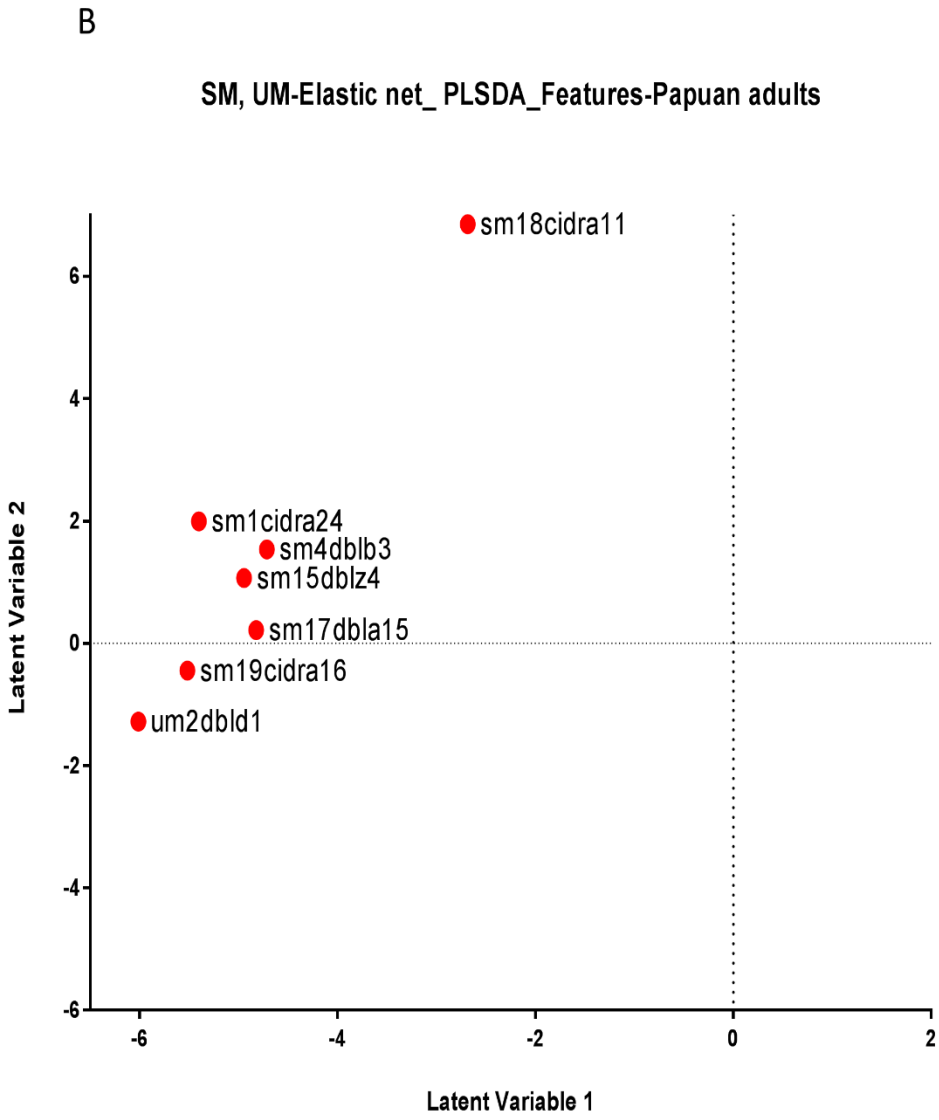

B
SM, UM-Elastic net_ PLSDA_Features-Papuan adults
sm18cidra11
sm1cidra24
sm4dblb3
sm15dblz4
sm17dbla15
sm19cidra16
um2dbld1
Latent Variable 2
Latent Variable 1
6
4
2
0
-2
-4
-6
-6
-4
-2
0
2

Figure 6.1 Relationships between antibody responses to PfEMP1 domains in Papuan individuals with severe and uncomplicated malaria.

Associations between the antibody responses against the PfEMP1 domains upregulated in severe or uncomplicated malaria using the elastic net, feature selection, PLSDA model are represented in this figure. X-axis represents the latent variable scores for the model in the first dimension analysis. Y-axis represents the latent variable scores for the model in the second dimension analysis. A: Individuals with severe malaria are represented by blue dots and individuals with uncomplicated malaria are represented in red dots. B: The PfEMP1 domains associated with antibody responses in individuals with uncomplicated malaria. SM, severe malaria; UM, uncomplicated malaria; PLSDA, Partial least squares discriminant analysis; LOOCV, leave one out cross validation; RMSEcv, root mean squared error of the cross validation; Var, variance; sqrtMSET, square root mean square error.

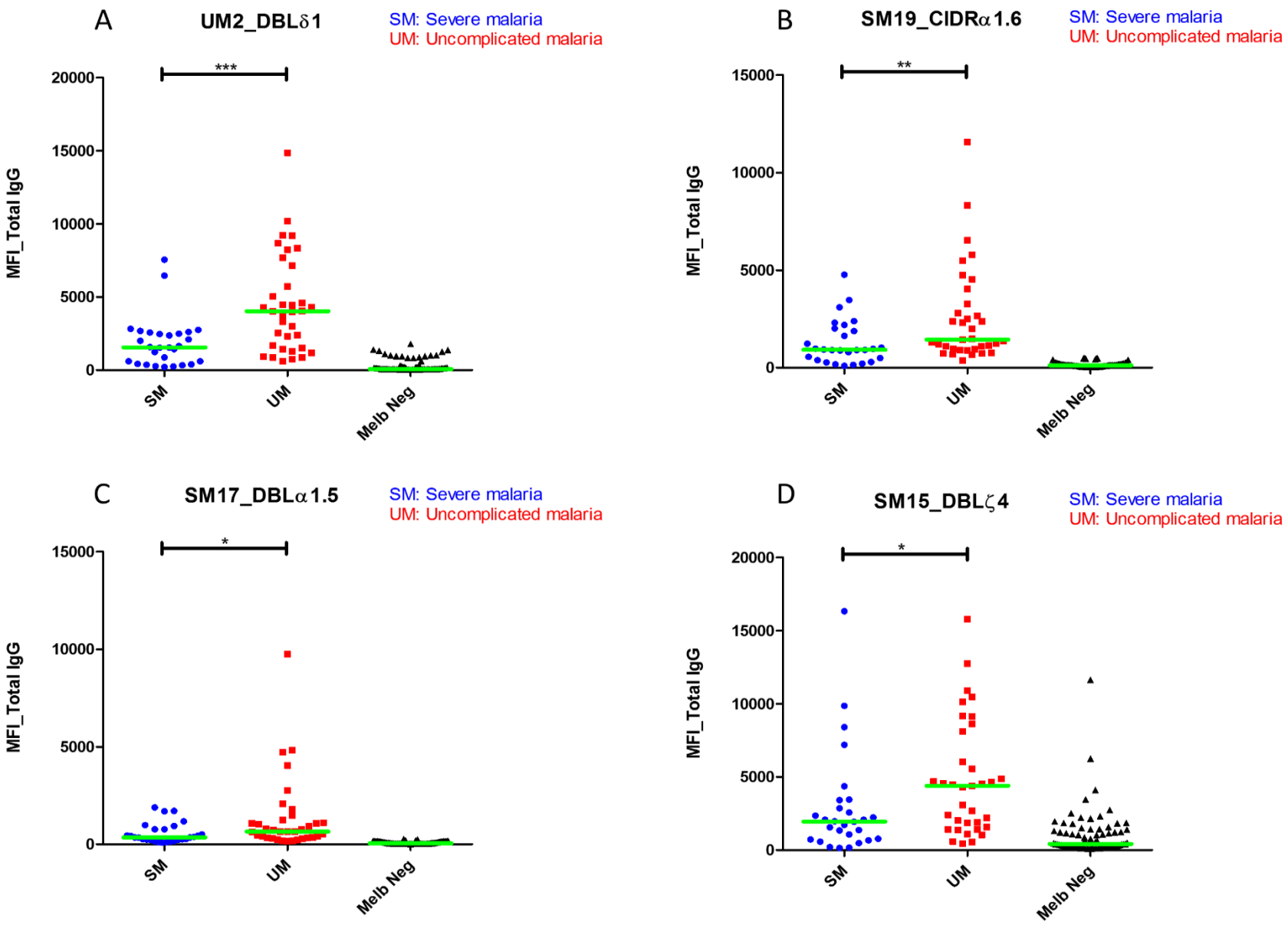
6.3.5 Antibody levels against individual PfEMP1 domains in Papuan individuals with severe or uncomplicated malaria

We first wanted to investigate the differences in antibody levels between individuals with severe malaria or uncomplicated malaria against the seven PfEMP1 domains that were shown to be responsible for the greatest variation in antibody responses (by severity) in the previous feature selection, PLSDA analyses (Figure 6.1B). We also wanted to determine if antibody levels differed by disease severity against the remaining PfEMP1 domains whose transcription was upregulated in severe and uncomplicated malaria but were not detected in the previous feature selection, PLSDA analysis. Machine learning models present multivariate statistical associations between our measured features. The current analysis helped us identify differences in antibody levels against the individual PfEMP1 domains associated with severe malaria or uncomplicated malaria in individuals with different disease severities. For all comparisons except antibody levels against tetanus toxin, which were higher in Melbourne negative controls (malaria naïve), antibody levels against the PfEMP1 domains in the Melbourne negative control individuals were significantly lower than those in participants with either type of malaria (statistics for these comparisons have not been provided).

6.3.5.1 Antibody levels against PfEMP1 domains most associated with variation in antibody responses

From figure 6.1B, the seven domains most associated with variation in antibody responses by severity are UM2-DBLδ1, upregulated in uncomplicated malaria, and SM19-CIDRα1.6, SM17-DBLα1.5, SM15-DBLζ4, SM4-DBLβ3, SM1-CIDRα2.4 and SM18-CIDRα1.1, upregulated in severe malaria.

We compared antibody levels in individuals with severe malaria and uncomplicated malaria against these domains. Participants with uncomplicated malaria had significantly higher antibody levels as compared to those with severe malaria against UM2-DBLδ1 ($p < 0.001$), SM19-CIDRα1.6 ($p = 0.007$), SM17-DBLα1.5 ($p = 0.013$), SM15-DBLζ4 ($p = 0.014$), SM4-DBLβ3 ($p = 0.023$) and SM1-CIDRα2.4 ($p = 0.015$) (Figure 6.2A-F). However, antibody levels were similar between the two disease severity groups for SM18-CIDRα1.1 ($p = 0.841$) (Figure 6.2G).


A
UM2_DBLδ1
SM: Severe malaria
UM: Uncomplicated malaria
***
MFI_Total IgG
20000
15000
10000
5000
0
SM
UM
MelbNeg
B
SM19_CIDRα1.6
SM: Severe malaria
UM: Uncomplicated malaria
**
MFI_Total IgG
15000
10000
5000
0
SM
UM
MelbNeg
C
SM17_DBLα1.5
SM: Severe malaria
UM: Uncomplicated malaria
*
MFI_Total IgG
15000
10000
5000
0
SM
UM
MelbNeg
D
SM15_DBLζ4
SM: Severe malaria
UM: Uncomplicated malaria
*
MFI_Total IgG
20000
15000
10000
5000
0
SM
UM
MelbNeg

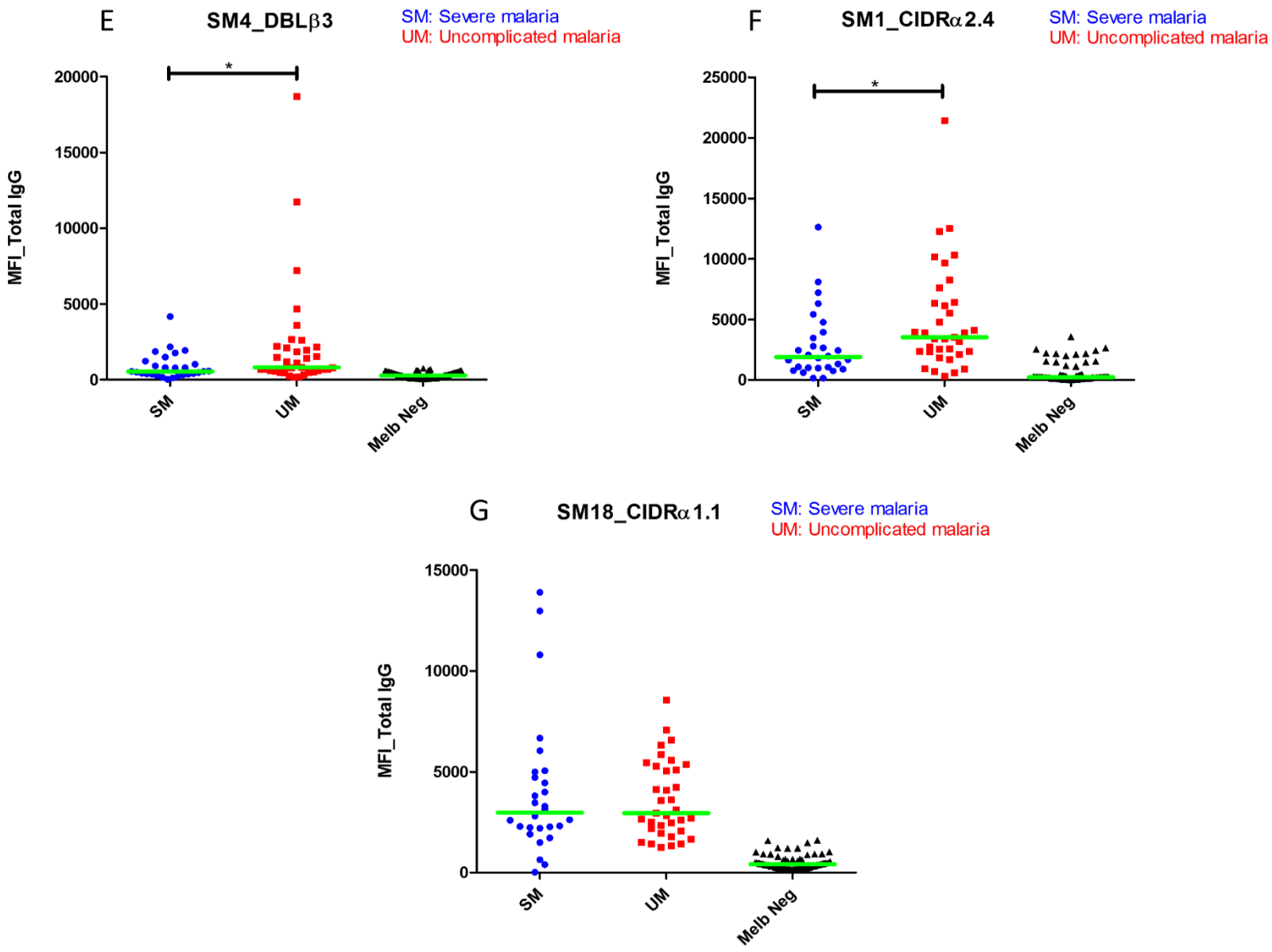
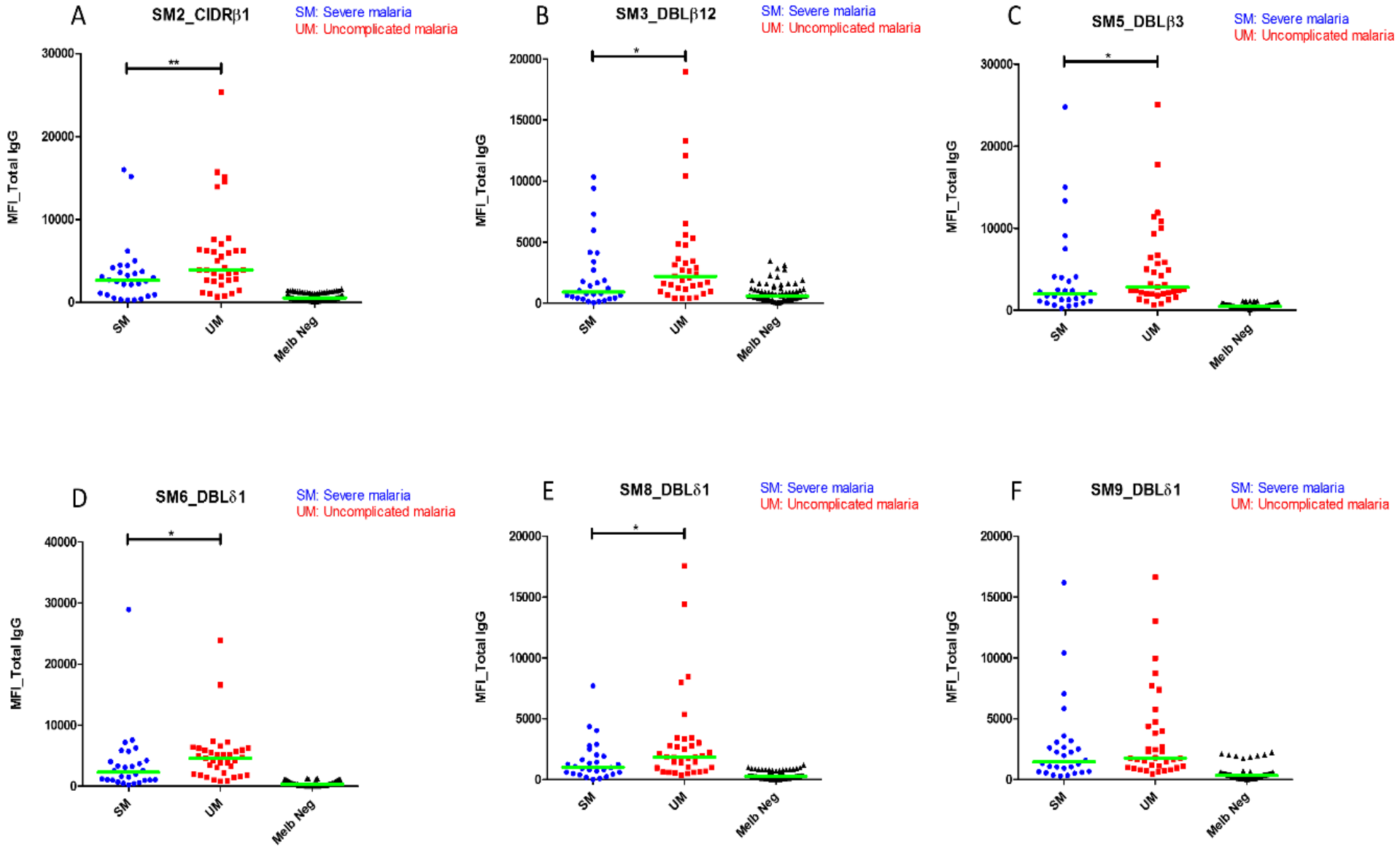

E
SM4_DBLβ3
SM: Severe malaria
UM: Uncomplicated malaria
MFI_Total IgG
20000
15000
10000
5000
0
*
SM
UM
Melb Neg
F
SM1_CIDRα2.4
SM: Severe malaria
UM: Uncomplicated malaria
MFI_Total IgG
25000
20000
15000
10000
5000
0
*
SM
UM
Melb Neg
G
SM18_CIDRα1.1
SM: Severe malaria
UM: Uncomplicated malaria
MFI_Total IgG
15000
10000
5000
0
SM
UM
Melb Neg

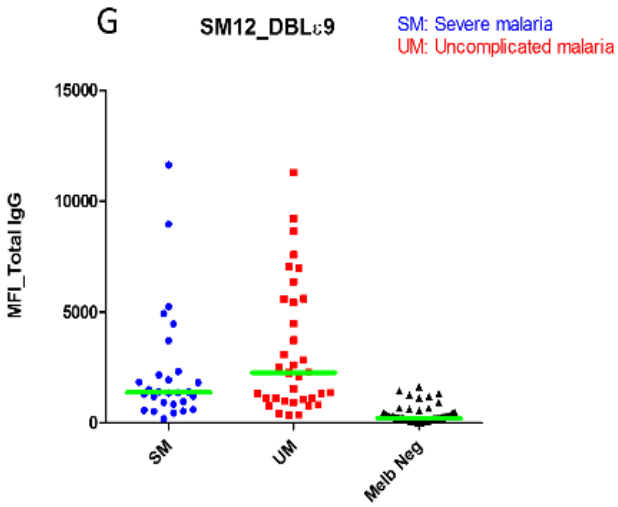
Figure 6.2 Antibody levels in Papuan individuals against PfEMP1 domains most associated with variation in antibody responses by severity.

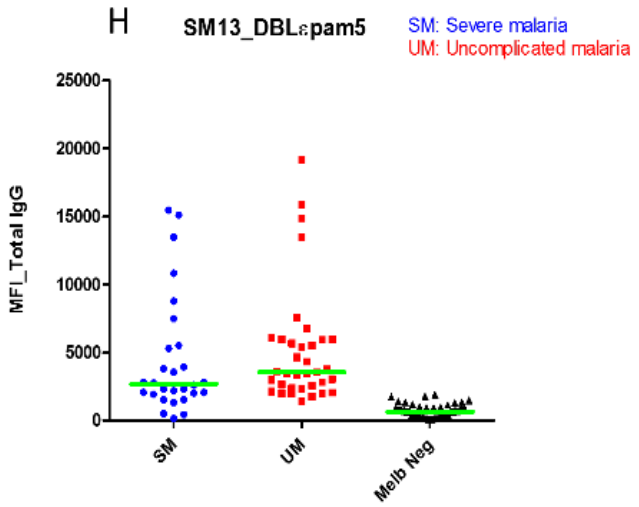
Using Wilcoxon Rank Sum test, individuals with severe malaria and uncomplicated malaria were compared. Y-axis represents the total IgG levels as median fluorescence intensity. Median antibody levels are presented as green horizontal bars for each disease severity group. Significant associations indicated by p-values: * < 0.05, ** < 0.01, *** <0.001, **** < 0.0001. Total number of participants in the severe malaria group, n = 28 and uncomplicated malaria group, n = 35. 77 antibody measurements from an average of 10 malaria naïve negative controls (from Melbourne) were also compared. Antibody levels were measured against the following domains, A: domain upregulated in uncomplicated malaria. B-G: domains upregulated in severe malaria. MFI, median fluorescence intensity; IgG, immunoglobulin G; UM, uncomplicated malaria; SM, severe malaria; Melb neg, malaria naïve negative controls from Melbourne.

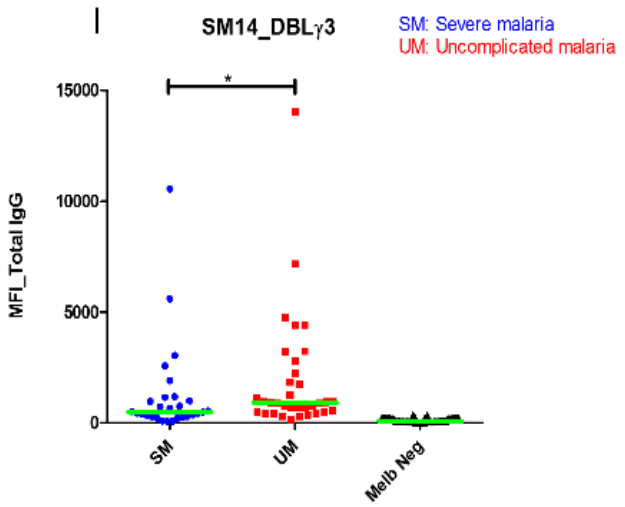
6.3.5.2 Antibody levels against the remaining PfEMP1 domains that were expressed at higher levels in severe than uncomplicated malaria but were not amongst the 7 PfEMP1 domains that contributed most to the variation in antibody responses

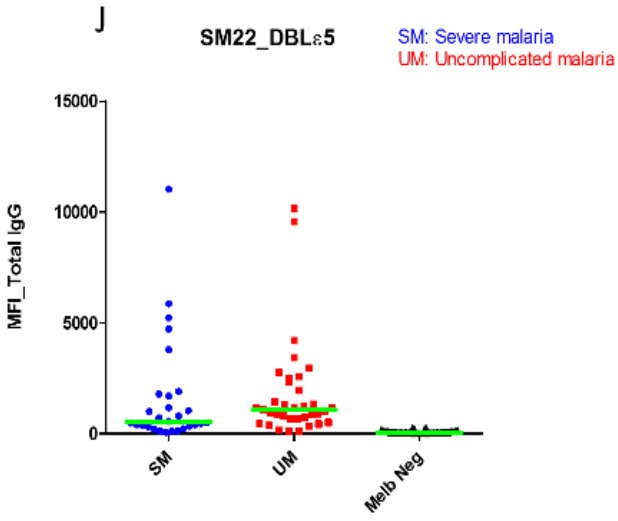
We next compared antibody levels against 16 other PfEMP1 domains, transcripts for which upregulated in severe malaria but were not identified in the previous feature selection, PLSDA analysis (Figure 6.1). Antibody levels were significantly higher in individuals with uncomplicated malaria compared to severe malaria against 10 out of 16 PfEMP1 domains: SM2-CIDRβ1 ($p = 0.008$), SM3-DBLβ12 ($p = 0.025$), SM5-DBLβ3 ($p = 0.039$), SM6-DBLδ1 ($p = 0.014$), SM8- DBLδ1 ($p = 0.024$), SM14-DBLγ3 ($p = 0.024$), SM24-DBLζ3 ($p = 0.029$), SM25-DBLβ13 ($p = 0.005$), SM26-CIDRγ12 ($p = 0.013$) and SM27-DBLδ7 ($p = 0.021$). The antibody levels did not differ significantly between the two disease severity groups for the other 6 PfEMP1 domains even though the trend was similar as observed above for most of the domains (Figure 6.3). These domains include: SM9-DBLδ1 ($p = 0.226$), SM12- DBLε9 ($p = 0.178$), SM13-DBLεpam5 ($p = 0.126$), SM22-DBLε5 ($p = 0.129$), SM11-DBLε3 ($p = 0.278$) and SM28-CIDRα2.6-DBLβ5 ($p = 0.516$).

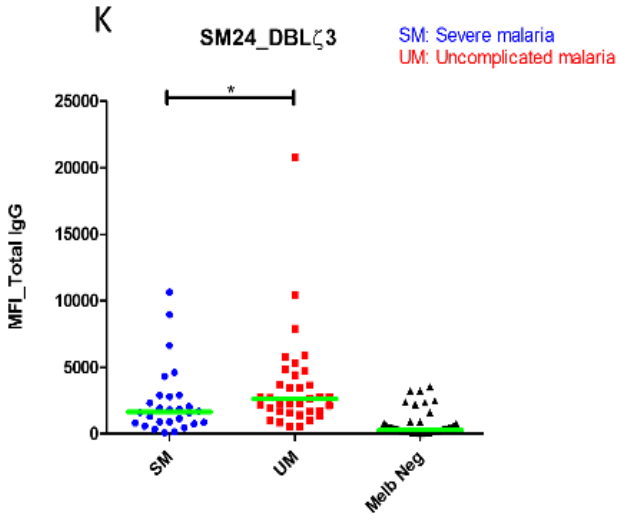

A
SM2_CIDRβ1
SM: Severe malaria
UM: Uncomplicated malaria
MFI_Total IgG
30000
20000
10000
0
**
SM
UM
Melb Neg
B
SM3_DBLβ12
SM: Severe malaria
UM: Uncomplicated malaria
MFI_Total IgG
20000
15000
10000
5000
0
*
SM
UM
Melb Neg
C
SM5_DBLβ3
SM: Severe malaria
UM: Uncomplicated malaria
MFI_Total IgG
30000
20000
10000
0
*
SM
UM
Melb Neg
D
SM6_DBLδ1
SM: Severe malaria
UM: Uncomplicated malaria
MFI_Total IgG
40000
30000
20000
10000
0
*
SM
UM
Melb Neg
E
SM8_DBLδ1
SM: Severe malaria
UM: Uncomplicated malaria
MFI_Total IgG
20000
15000
10000
5000
0
*
SM
UM
Melb Neg
F
SM9_DBLδ1
SM: Severe malaria
UM: Uncomplicated malaria
MFI_Total IgG
20000
15000
10000
5000
0
SM
UM
Melb Neg

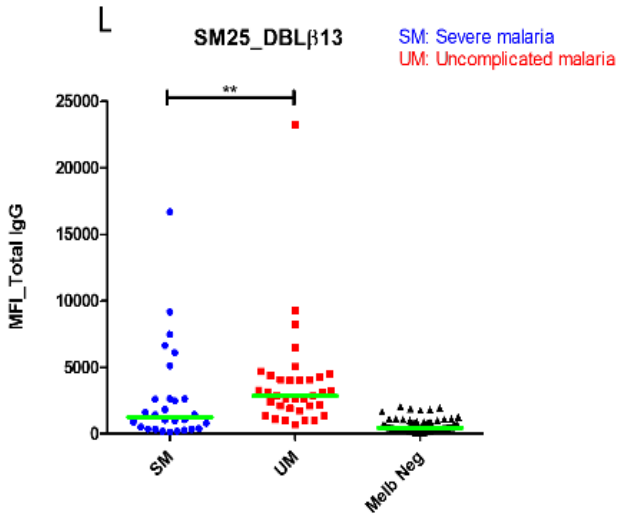

G
SM12_DBLε9
SM: Severe malaria
UM: Uncomplicated malaria
MFI_Total IgG
15000
10000
5000
0
SM
UM
Melb Neg


H
SM13_DBLεpam5
SM: Severe malaria
UM: Uncomplicated malaria
MFI_Total IgG
25000
20000
15000
10000
5000
0
SM
UM
Melb Neg


I
SM14_DBLγ3
SM: Severe malaria
UM: Uncomplicated malaria
*
MFI_Total IgG
15000
10000
5000
0
SM
UM
Melb Neg


J
SM22_DBLε5
SM: Severe malaria
UM: Uncomplicated malaria
MFI_Total IgG
15000
10000
5000
0
SM
UM
Melb Neg


K
SM24_DBLζ3
SM: Severe malaria
UM: Uncomplicated malaria
*
MFI_Total IgG
25000
20000
15000
10000
5000
0
SM
UM
Melb Neg


L
SM25_DBLβ13
SM: Severe malaria
UM: Uncomplicated malaria
**
MFI_Total IgG
25000
20000
15000
10000
5000
0
SM
UM
Melb Neg

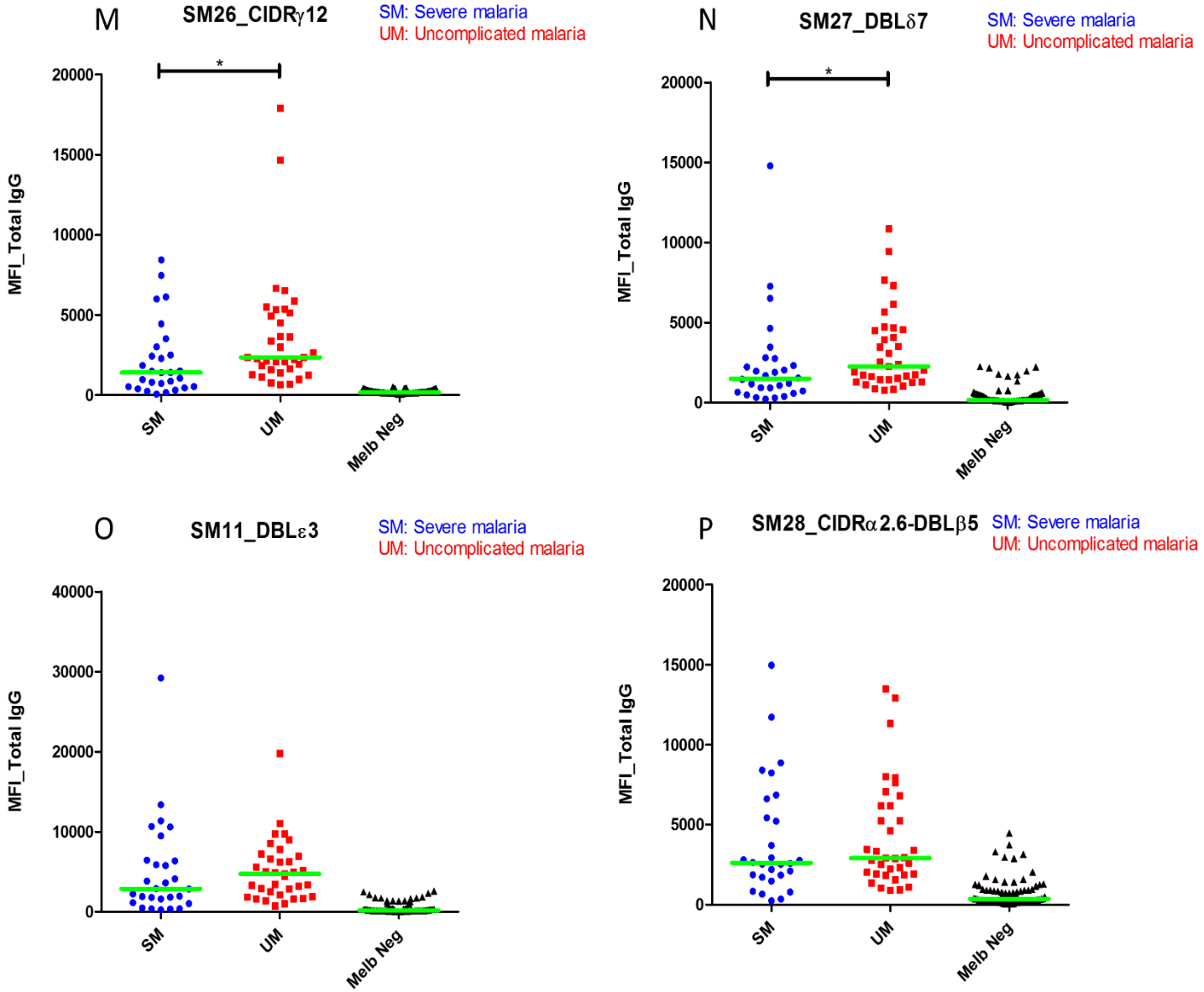
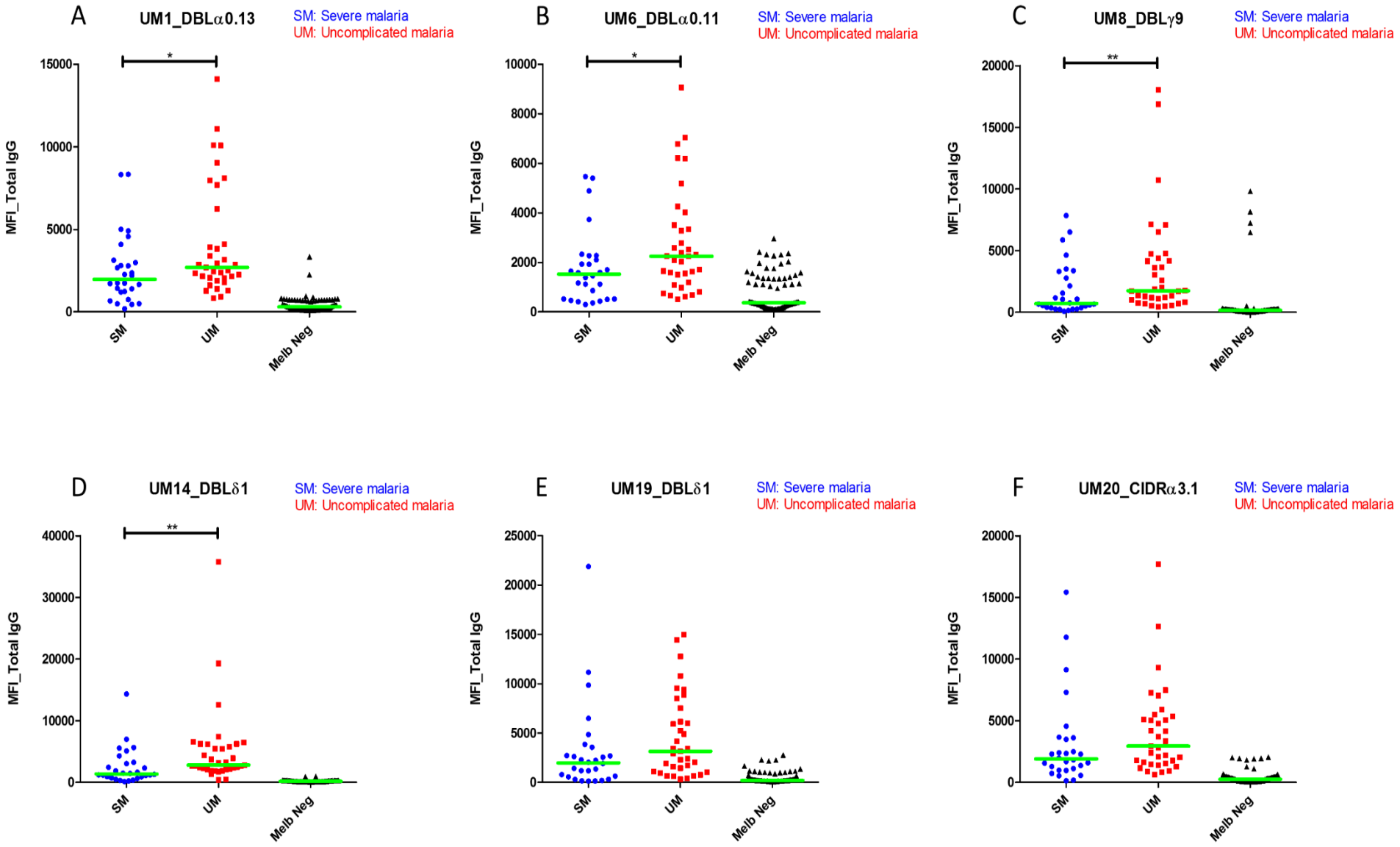

M
SM26_CIDRγ12
SM: Severe malaria
UM: Uncomplicated malaria
MFI_Total IgG
20000
15000
10000
5000
0
*
SM
UM
Melb Neg
N
SM27_DBLδ7
SM: Severe malaria
UM: Uncomplicated malaria
MFI_Total IgG
20000
15000
10000
5000
0
*
SM
UM
Melb Neg
O
SM11_DBLε3
SM: Severe malaria
UM: Uncomplicated malaria
MFI_Total IgG
40000
30000
20000
10000
0
SM
UM
Melb Neg
P
SM28_CIDRα2.6-DBLβ5
SM: Severe malaria
UM: Uncomplicated malaria
MFI_Total IgG
20000
15000
10000
5000
0
SM
UM
Melb Neg

Figure 6.3 Antibody levels in Papuan individuals against the remaining PfEMP1 domains upregulated in severe malaria.

The PfEMP1 domains tested here are the ones whose transcripts were upregulated in severe malaria compared to uncomplicated malaria but were not amongst the 7 PfEMP1 domains that contributed most to the variation in antibody responses by severity**.** Using Wilcoxon Rank Sum test, individuals with severe malaria and uncomplicated malaria were compared. Y-axis represents the total IgG levels as median fluorescence intensity. Median antibody levels are presented as green horizontal bars for each disease severity group. Significant associations indicated by p-values: * < 0.05, ** < 0.01, *** <0.001, **** < 0.0001. Total number of participants in the severe malaria group, n = 28 and uncomplicated malaria group, n = 35. 77 antibody measurements from an average of 10 malaria naïve negative controls (from Melbourne) were also compared. A-P: Antibody levels were measured against PfEMP1 domains upregulated in patients with severe malaria. MFI, median fluorescence intensity; IgG, immunoglobulin G; SM, severe malaria; UM, uncomplicated malaria; Melb neg, malaria naïve negative controls from Melbourne.

6.3.5.3 Antibody levels against the remaining PfEMP1 domains that were expressed at higher levels in uncomplicated than severe malaria but were not amongst the 7 PfEMP1 domains that contributed most to the variation in antibody responses

Antibody levels were compared between individuals with severe malaria and uncomplicated malaria against 9 other PfEMP1 domains whose transcription was upregulated in individuals with uncomplicated malaria (but were not amongst the 7 domains that contributed most to the variation in antibody responses by severity in the previous feature selection, PLSDA analysis, figure 6.1). Significant differences in antibody levels between the two disease severity groups were found in 6 out of the 9 domains. The antibody levels were higher in participants with uncomplicated malaria as compared to severe malaria against: UM1-DBLα0.13 ($p = 0.032$), UM6-DBLα0.11 ($p = 0.021$), UM8-DBLγ9 ($p = 0.006$), UM14-DBL δ1 ($p = 0.001$), UM43-DBLpam2 ($p = 0.011$) and UM45-CIDRα1.7 ($p = 0.049$). For the remaining 3 PfEMP1 domains, UM19-DBL δ1 ($p = 0.055$), UM20-CIDRα3.1 ($p = 0.078$) and UM21-DBLα0.9 ($p = 0.078$), although the antibody levels in the uncomplicated malaria group were higher than the severe malaria group, these differences were not significant (Figure 6.4).


A
UM1_DBLα0.13
SM: Severe malaria
UM: Uncomplicated malaria
MFI_Total IgG
15000
10000
5000
0
*
SM
UM
Melb Neg
B
UM6_DBLα0.11
SM: Severe malaria
UM: Uncomplicated malaria
MFI_Total IgG
10000
8000
6000
4000
2000
0
*
SM
UM
Melb Neg
C
UM8_DBLγ9
SM: Severe malaria
UM: Uncomplicated malaria
MFI_Total IgG
20000
15000
10000
5000
0
**
SM
UM
Melb Neg
D
UM14_DBLδ1
SM: Severe malaria
UM: Uncomplicated malaria
MFI_Total IgG
40000
30000
20000
10000
0
**
SM
UM
Melb Neg
E
UM19_DBLδ1
SM: Severe malaria
UM: Uncomplicated malaria
MFI_Total IgG
25000
20000
15000
10000
5000
0
SM
UM
Melb Neg
F
UM20_CIDRα3.1
SM: Severe malaria
UM: Uncomplicated malaria
MFI_Total IgG
20000
15000
10000
5000
0
SM
UM
Melb Neg

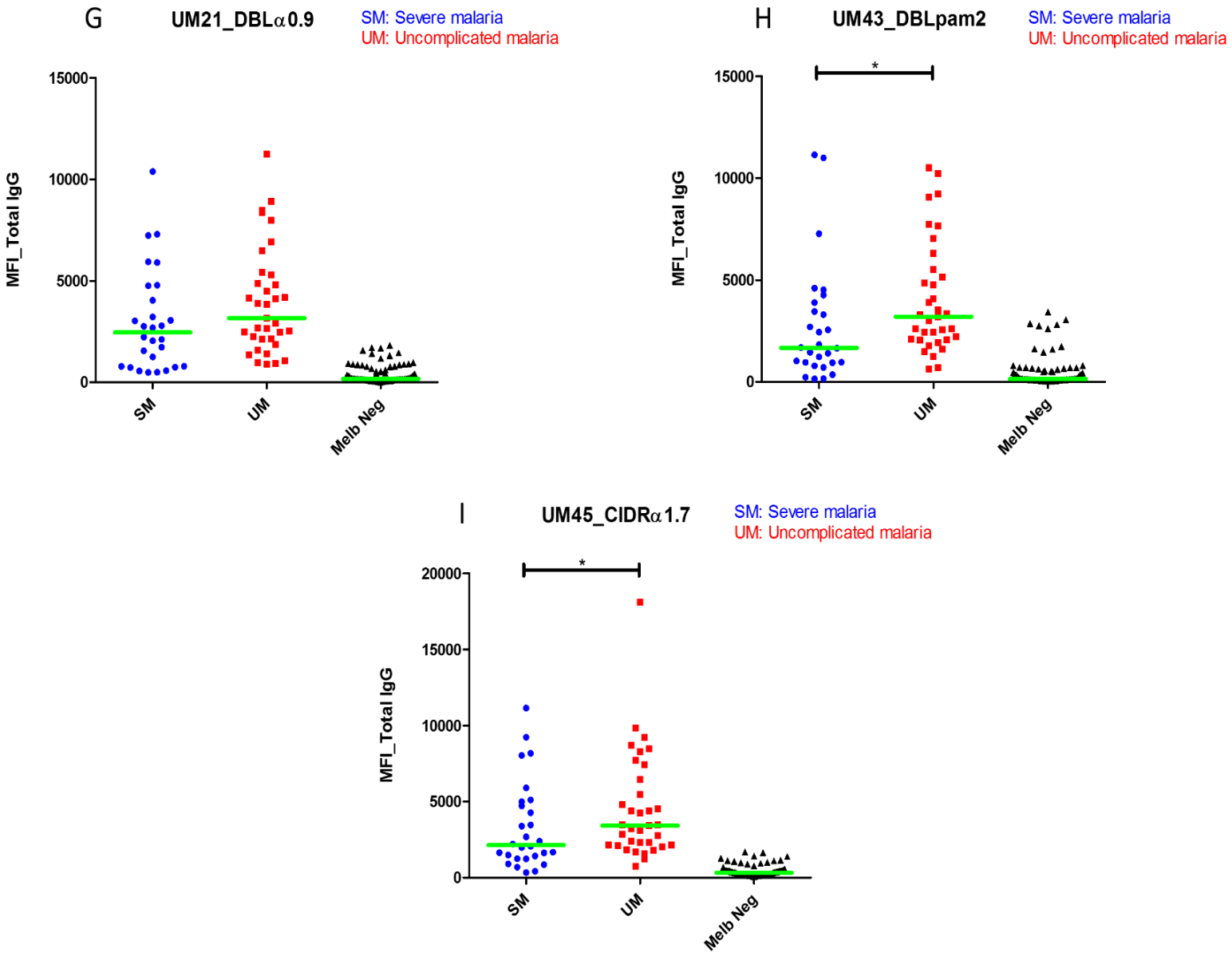

G
UM21_DBLα0.9
SM: Severe malaria
UM: Uncomplicated malaria
MFI_Total IgG
15000
10000
5000
0
SM
UM
Melb Neg
H
UM43_DBLpam2
SM: Severe malaria
UM: Uncomplicated malaria
*
MFI_Total IgG
15000
10000
5000
0
SM
UM
Melb Neg
I
UM45_CIDRα1.7
SM: Severe malaria
UM: Uncomplicated malaria
*
MFI_Total IgG
20000
15000
10000
5000
0
SM
UM
Melb Neg

Figure 6.4 Antibody levels in Papuan individuals against the remaining PfEMP1 domains upregulated in uncomplicated malaria.
The PfEMP1 domains tested here are the ones whose transcripts were upregulated in uncomplicated malaria compared to severe malaria but were not amongst the 7 PfEMP1 domains that contributed most to the variation in antibody responses by severity**.** Using Wilcoxon Rank Sum test, individuals with severe malaria and uncomplicated malaria were compared. Y-axis represents the total IgG levels as median fluorescence intensity. Median antibody levels are presented as green horizontal bars for each disease severity group. Significant associations indicated by p-values: * < 0.05, ** < 0.01, *** <0.001, **** < 0.0001. Total number of participants in the severe malaria group, n = 28 and uncomplicated malaria group, n = 35. 77 antibody measurements from an average of 10 malaria naïve negative controls (from Melbourne) were also compared. A-I: Antibody levels were measured against PfEMP1 domains upregulated in patients with uncomplicated malaria. MFI, median fluorescence intensity; IgG, immunoglobulin G; SM, severe malaria; UM, uncomplicated malaria; Melb neg, malaria naïve negative controls from Melbourne.

6.3.5.4 Antibody levels against control proteins

Antibody levels were also compared between individuals with severe or uncomplicated malaria against 2 *P. falciparum* control proteins, AMA-1 and CSP and one tetanus antigen. The control proteins were tested twice while measuring antibodies against 32 PfEMP1 domains. No significant differences in the antibody levels were observed on comparing the two groups (except for CSP protein in one case, where individuals with uncomplicated malaria showed weak evidence for having higher antibody levels as compared to severe malaria participants, $p = 0.044$) (Figure 6.5).

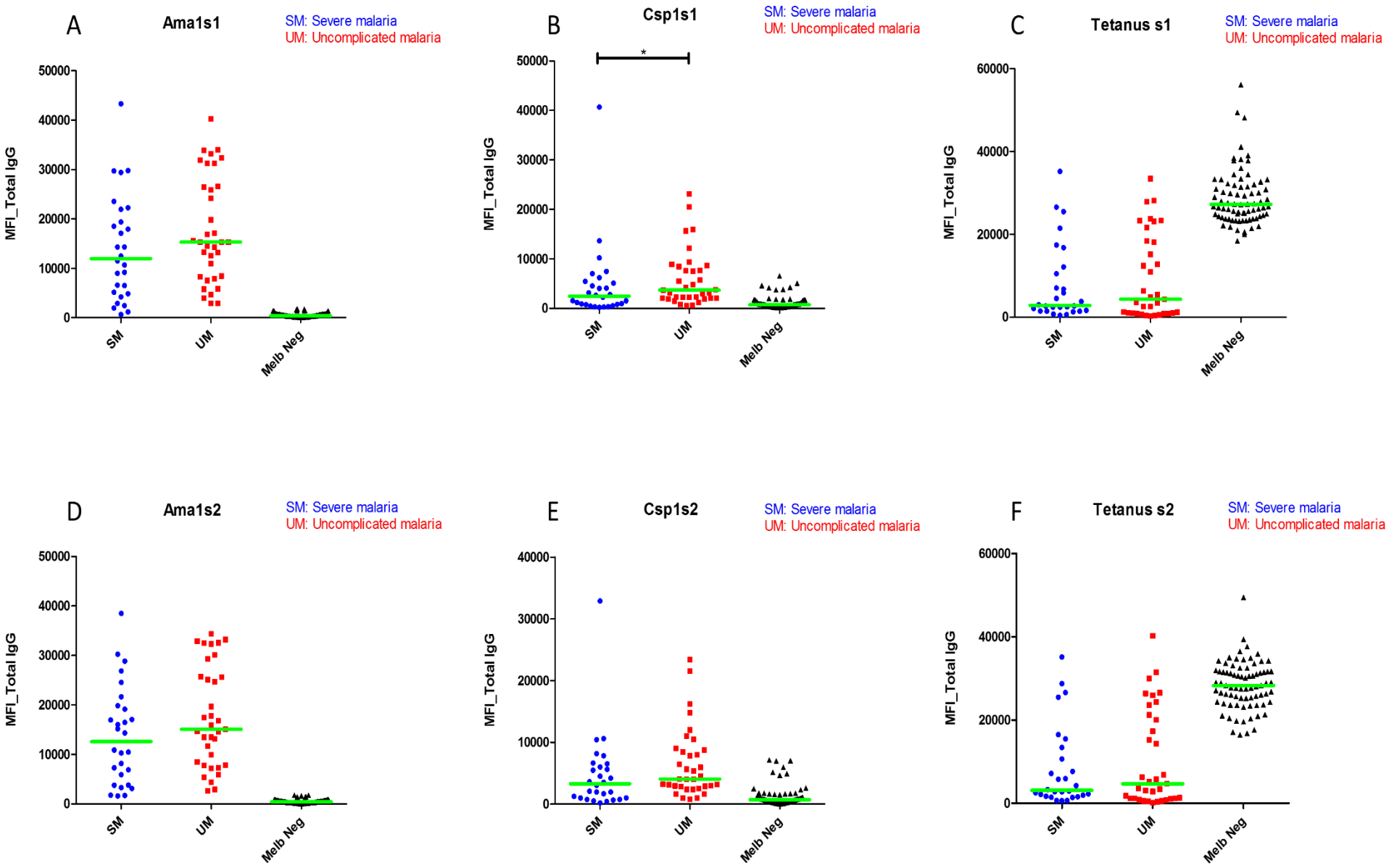

A
Ama1s1
SM: Severe malaria
UM: Uncomplicated malaria
MFI_Total IgG
50000
40000
30000
20000
10000
0
SM
UM
Melb Neg
B
Csp1s1
SM: Severe malaria
UM: Uncomplicated malaria
*
MFI_Total IgG
50000
40000
30000
20000
10000
0
SM
UM
Melb Neg
C
Tetanus s1
SM: Severe malaria
UM: Uncomplicated malaria
MFI_Total IgG
60000
40000
20000
0
SM
UM
Melb Neg
D
Ama1s2
SM: Severe malaria
UM: Uncomplicated malaria
MFI_Total IgG
50000
40000
30000
20000
10000
0
SM
UM
Melb Neg
E
Csp1s2
SM: Severe malaria
UM: Uncomplicated malaria
MFI_Total IgG
40000
30000
20000
10000
0
SM
UM
Melb Neg
F
Tetanus s2
SM: Severe malaria
UM: Uncomplicated malaria
MFI_Total IgG
60000
40000
20000
0
SM
UM
Melb Neg

Figure 6.5 Antibody levels in Papuan individuals against control proteins.

Using Wilcoxon Rank Sum test, individuals with severe malaria and uncomplicated malaria were compared. Y-axis represents the total IgG levels as median fluorescence intensity. Median antibody levels are presented as green horizontal bars for each disease severity group. Significant associations indicated by p-values: * < 0.05, ** < 0.01, *** <0.001, **** < 0.0001. Total number of participants in the severe malaria group, n = 28 and uncomplicated malaria group, n = 35. 77 antibody measurements from an average of 10 malaria naïve negative controls (from Melbourne) were also compared. A-F: Antibody levels were measured against three control proteins (and were tested twice). MFI, median fluorescence intensity; IgG, immunoglobulin G; SM, severe malaria; UM, uncomplicated malaria; Melb neg, malaria naïve negative controls from Melbourne.

6.3.6 Identifying relationships between antibody responses to PfEMP1 domains in Malawian children with cerebral and uncomplicated malaria at hospital presentation and during follow-up

The PfEMP1 domains tested for antibody levels in children from Malawi are domains whose transcription was upregulated in severe or uncomplicated malaria cases in Papua, Indonesia. One of the key questions is whether antibodies from Malawian children recognise the domains upregulated in Papuan individuals and if the antibody levels differ by disease severity. This type of analysis was carried out by Dr. Amy Chung and Mr. Timon Damelang.

In order to determine relationships between antibody responses in children with cerebral malaria and uncomplicated malaria at presentation and follow-up, a machine learning model using PCA (unsupervised approach) was carried out. Signatures of antibody responses against 22 PfEMP1 domains upregulated in severe malaria cases, 10 domains upregulated in uncomplicated malaria and 2 *P. falciparum* control proteins were determined.

Results are presented in figure 6.6. Each dot on the PCA plot represents the multivariate analysis of all antibody values against all 32 PfEMP1 domains (and 2 control proteins) in each child with their malaria diagnostics indicated (purple dots = cerebral acute, green dots = cerebral follow-up, orange dots = uncomplicated acute, black dots = uncomplicated follow-up) along two dimensions. X-axis represents the PCA score for the model in the first dimension while on Y-axis we have the PCA score for the second dimension analysis. Along both dimensions (axes), we did not observe distinct clustering of antibody responses against the 32 PfEMP1 domains tested between the four disease severity groups (total variance = 40.1%) (Figure 6.6A). Most of the separation seems to occur along the first dimension (X-axis, variance = 33.9%), with clustering along the second dimension (Y-axis, variance = 6.1%) being much lower. Based on analysis along the first dimension (X-axis) (Figure 6.6A), children with cerebral acute malaria (represented by purple dots) cluster somewhat separately from the children with uncomplicated acute malaria (represented by orange dots). The PfEMP1 domains (Figure 6.6B) did not segregate into clusters defined by varying degrees of reactivity with patient groups. Similarly, antibody levels in children with uncomplicated malaria at acute time point (orange dots) clustered slightly separately to antibody levels in the same children during follow-up (black dots) (Figure 6.6A). Antibody responses in children with cerebral or uncomplicated malaria at follow-up (represented by green and black dots respectively) were quite overlapping and lacked distinct clustering (Figure 6.6A). Similarly, antibody

responses in children with cerebral malaria at acute and follow-up time points (purple and green dots respectively) did not seem to cluster separately (Figure 6.6A).

To further explore differences in antibody responses between children with cerebral malaria and uncomplicated malaria at presentation, a PLSDA model without any feature selection was applied (Figure 6.7). X and Y-axes represent the latent variable scores for first and second dimension analysis respectively and each dot is a multivariate analysis of all the antibody responses for each individual targeted against all the PfEMP1 domains tested. Antibody responses in children with cerebral malaria, represented by purple dots, clustered somewhat separately as compared to antibody responses in children with uncomplicated malaria (orange dots) (LOOCV = 56.1%, combined variance = 41.6%) (Figure 6.7A). The combined variance represents the variance along the X-axis and Y-axis (first and second dimension analysis). Most of the clustering seemed to occur along the first dimension (X-axis), with not much clustering along the second dimension analysis (Y-axis). Based on the position of the features along X- axis (Figure 6.7B) in relation to clustering observed in figure 6.7A, the variation in antibody response was due to a greater overall response from children with cerebral acute malaria (purple dots on negative X-axis, variance = 36.8%) to the entire range of PfEMP1 domains tested regardless of whether the domains were upregulated in severe or uncomplicated malaria. We also applied the PLSDA model without any feature selection to assess differences in antibody responses in plasma collected from children with uncomplicated malaria at acute (orange dots) and follow-up time point (black dots) (Figure 6.8). The antibody responses in the two groups were mostly overlapping, with only a slight separation observed (LOOCV = 53.2%. Combined variance = 38.9%) (Figure 6.8A). These differences in antibody responses were also mainly along the first dimension analysis (X-axis, variance = 33.1%) (Figure 6.8A) and seemed to be driven by higher responses overall by uncomplicated follow-up cases to all of the PfEMP1 domains tested, regardless of whether the domains were upregulated in severe or uncomplicated malaria as indicated by the positive scores for all PfEMP1 features in the first dimension of figure 6.8B.

Thus, the antibody responses to the PfEMP1 domains in children from all the different severity groups did not cluster distinctly. Further comparisons in the antibody responses between cerebral and uncomplicated acute or uncomplicated acute and follow-up groups only showed slight clustering of antibody responses by severity (with the responses to PfEMP1 domains mainly overlapping by severity).

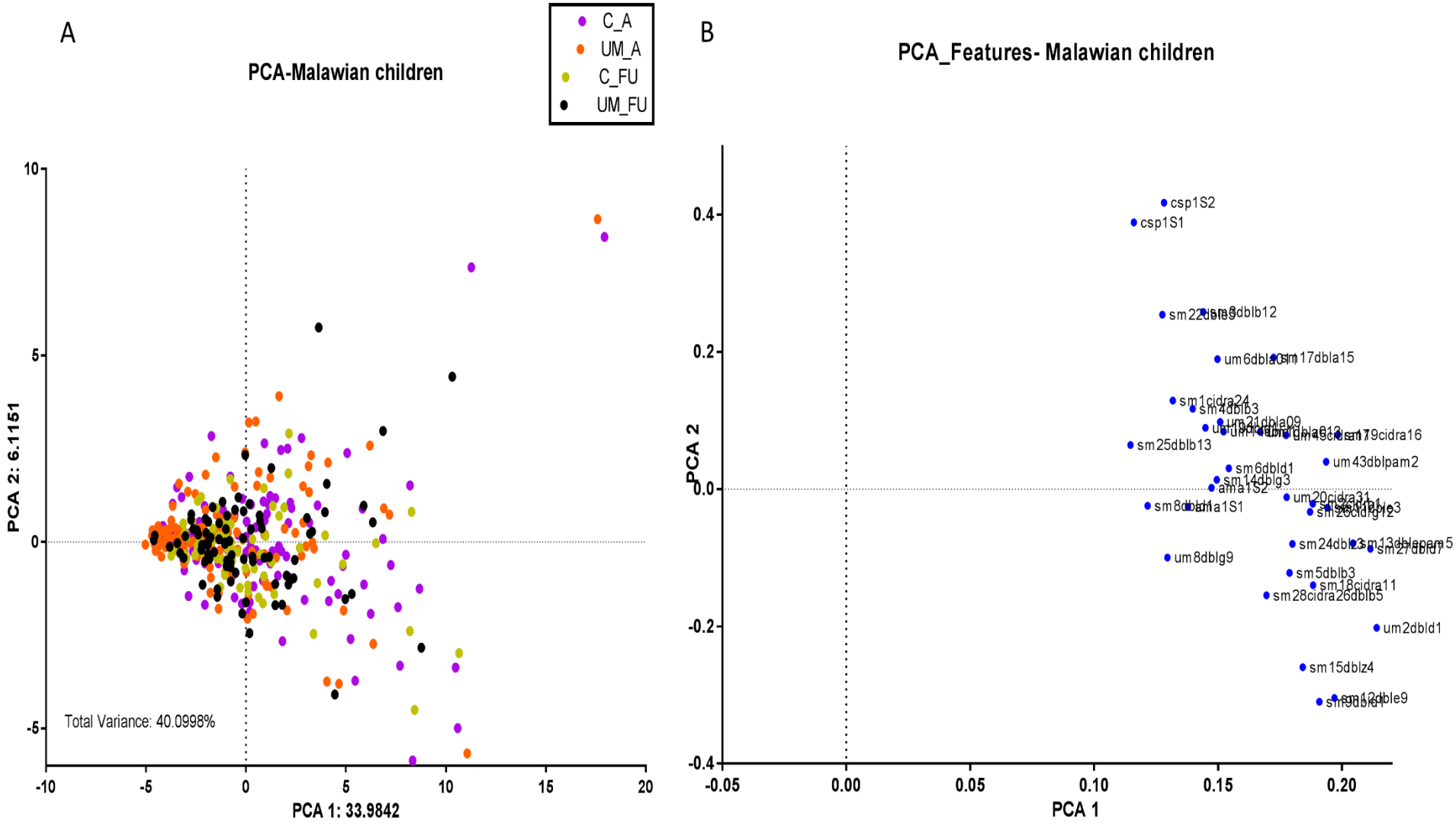

A
PCA-Malawian children
C_A
UM_A
C_FU
UM_FU
PCA 2: 6.1151
PCA 1: 33.9842
Total Variance: 40.0998%
B
PCA_Features- Malawian children
PCA 2
PCA 1
csp1S2
csp1S1
um6dblb3
sm17dbla15
sm1cidra24
sm25dblb13
sm6dbld1
um43dblpam2
sm14dblg3
ama1S2
um20cidra31
sm8dblb1
ama1S1
sm24dblb3
sm13dblapam5
sm27dbld7
um8dblg9
sm5dblb3
sm18cidra11
sm28cidra26dblb5
um2dbld1
sm15dblz4
sm12dble9

Figure 6.6 Relationships between antibody responses to PfEMP1 domains in Malawian children with cerebral and uncomplicated malaria at acute and follow-up period.

Associations between the antibody responses against the PfEMP1 domains upregulated in severe or uncomplicated malaria using the PCA are represented in this figure. X-axis represents the PCA scores for the model in the first dimension analysis. Y-axis represents the PCA scores for the model in the second dimension analysis. A: Individuals with cerebral acute malaria are represented by purple dots while follow-up children are presented as green dots; children with uncomplicated acute malaria are represented as orange dots while follow-up children are presented in black dots. B: The PfEMP1 domains associated with antibody responses in children with malaria. C_A, cerebral acute; UM_A, uncomplicated malaria acute; C_FU, cerebral follow-up; UM_FU, uncomplicated malaria follow-up; SM, severe malaria; UM, uncomplicated malaria; PCA, principal component analysis.

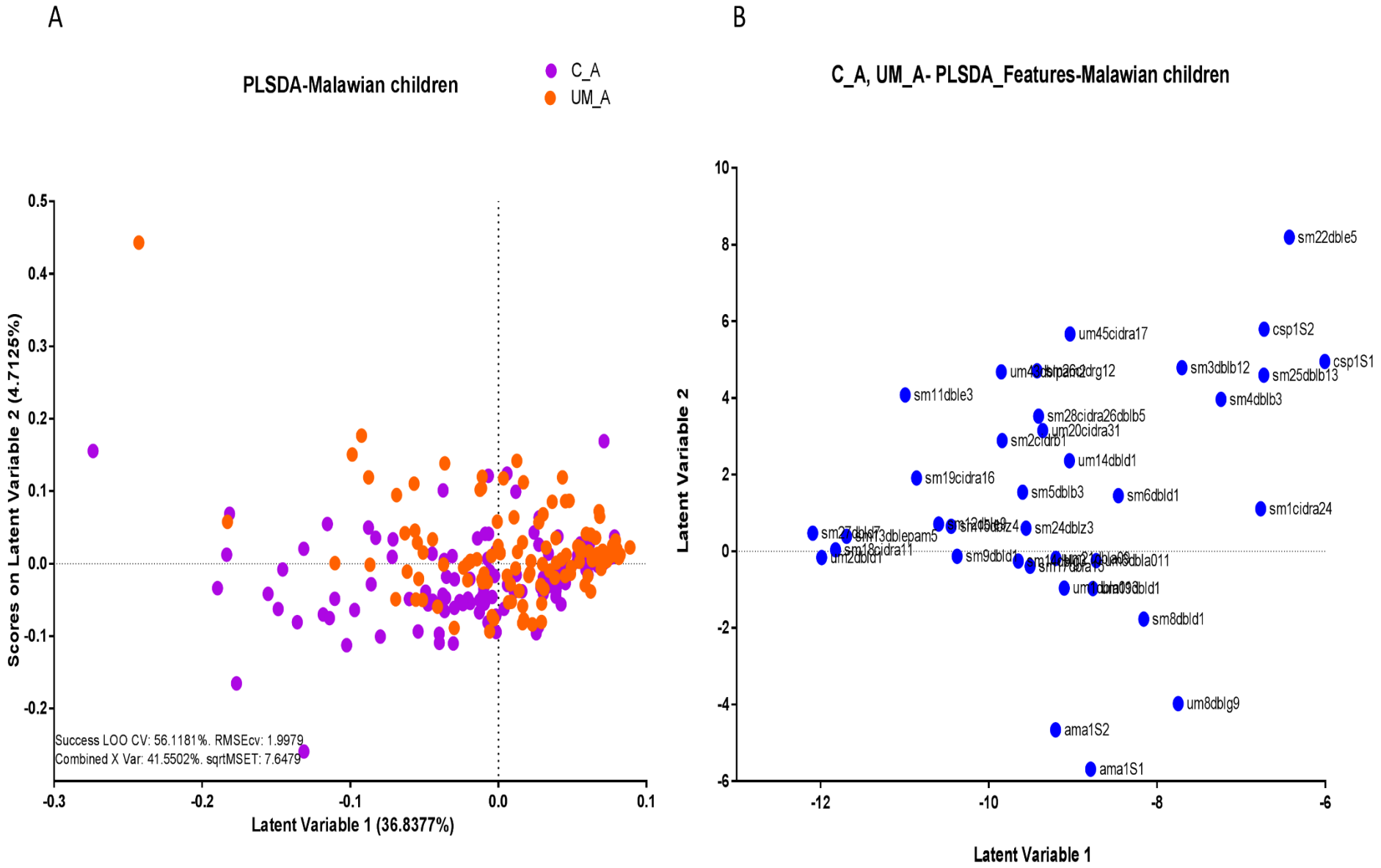
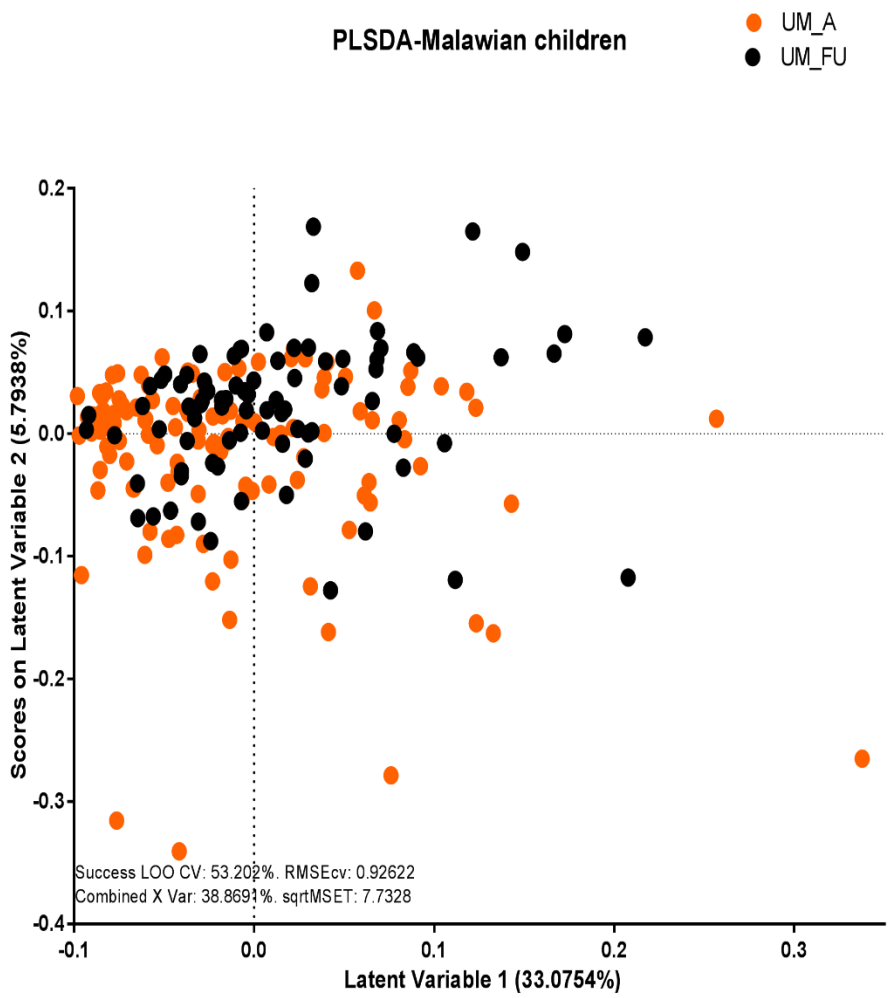

A
PLSDA-Malawian children
C_A
UM_A
Scores on Latent Variable 2 (4.7125%)
Latent Variable 1 (36.8377%)
Success LOO CV: 56.1181%. RMSEcv: 1.9979
Combined X Var: 41.5502%. sqrtMSET: 7.6479
B
C_A, UM_A- PLSDA_Features-Malawian children
Latent Variable 2
Latent Variable 1
sm22dble5
um45cidra17
csp1S2
csp1S1
sm3dblb12
sm25dblb13
sm11dble3
sm4dblb3
sm28cidra26dblb5
um20cidra31
sm2cidrb1
um14dbld1
sm19cidra16
sm5dblb3
sm6dbld1
sm1cidra24
sm24dblz3
sm13dblepam5
sm18cidra11
um2dbld1
sm9dbld1
sm8dbld1
um8dblg9
ama1S2
ama1S1

Figure 6.7 Relationships between antibody responses to PfEMP1 domains in Malawian children with cerebral and uncomplicated malaria at acute time point.

Using the PLSDA model, associations between the antibody responses against the PfEMP1 domains upregulated in severe or uncomplicated acute malaria are represented. X-axis represents the latent variable scores for the model in the first dimension analysis. Y-axis represents the latent variable scores for the model in the second dimension analysis. A: Children with cerebral acute malaria are represented by purple dots and children with uncomplicated acute malaria are represented in orange dots. B: The PfEMP1 domains associated with antibody responses in children with cerebral or uncomplicated acute malaria. C_A, cerebral acute; UM_A, uncomplicated malaria acute; PLSDA, Partial least squares discriminant analysis; SM, severe malaria; UM, uncomplicated malaria; LOOCV, leave one out cross validation; RMSEcv, root mean squared error of the cross validation; Var, variance; sqrtMSET, square root mean square error.

A


PLSDA-Malawian children
UM_A
UM_FU
Scores on Latent Variable 2 (5.7938%)
Latent Variable 1 (33.0754%)
Success LOO CV: 53.202%. RMSEcv: 0.92622
Combined X Var: 38.8691%. sqrtMSET: 7.7328

B

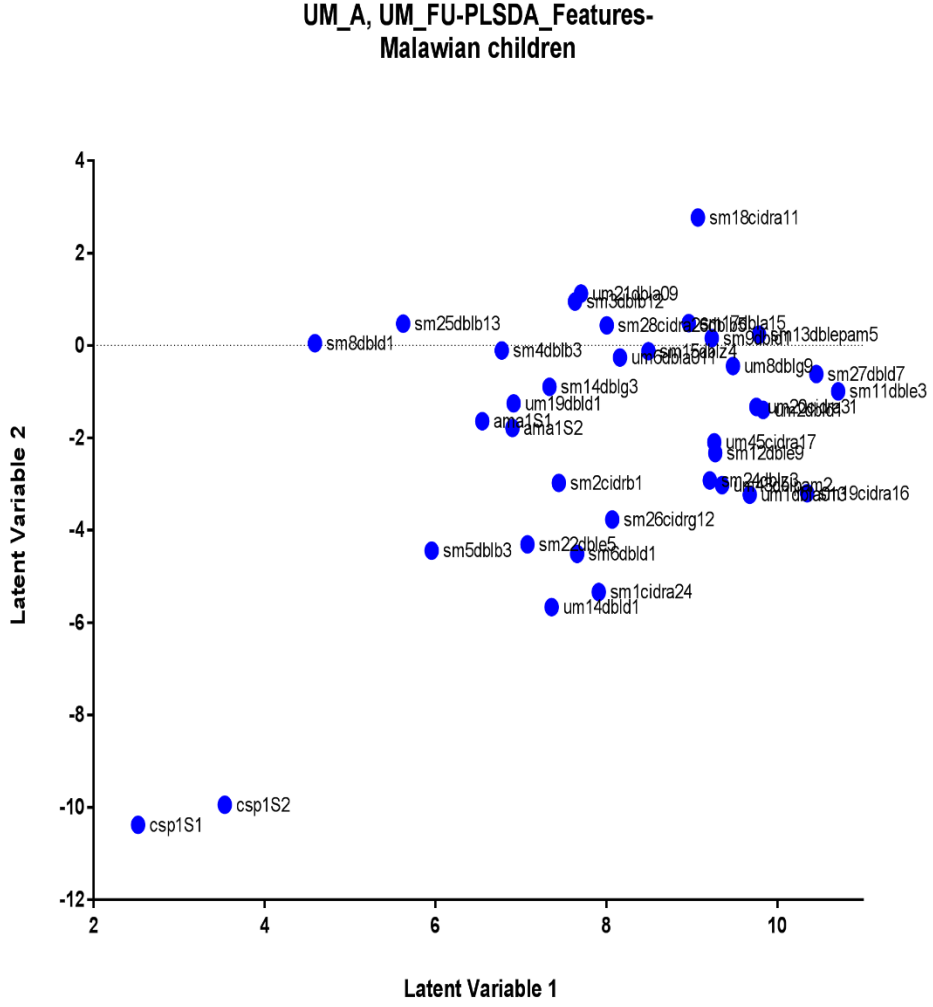

UM_A, UM_FU-PLSDA_Features-
Malawian children
Latent Variable 2
Latent Variable 1
sm18cidra11
sm25dblb13
sm8dbld1
sm4dblb3
sm14dblg3
um19dbld1
ama1S1
ama1S2
sm2cidrb1
sm26cidrg12
sm5dblb3
sm6dbld1
sm1cidra24
um14dbld1
csp1S1
csp1S2
um8dblg9
sm27dbld7
sm11dble3
um45cidra17
sm12dble9
sm13dblepam5

Figure 6.8 Relationships between antibody responses to PfEMP1 domains in Malawian children with uncomplicated malaria at acute and follow-up time points

Using the PLSDA model, associations between the antibody responses against the PfEMP1 domains upregulated in severe or uncomplicated acute malaria are represented. X-axis represents the latent variable scores for the model in the first dimension analysis. Y-axis represents the latent variable scores for the model in the second dimension analysis. A: Children with uncomplicated acute malaria are represented by orange dots and children with uncomplicated malaria during follow-up are represented in black dots. B: The PfEMP1 domains associated with antibody responses in children with uncomplicated malaria. UM_A, uncomplicated malaria acute; UM_FU, uncomplicated malaria at follow-up; PLSDA, Partial least squares discriminant analysis; SM, severe malaria; UM, uncomplicated malaria; LOOCV, leave one out cross validation; RMSEcv, root mean squared error of the cross validation; Var, variance; sqrtMSET, square root mean square error.

6.3.7 Antibody levels against PfEMP1 domains in Malawian children with cerebral or uncomplicated malaria at presentation and during follow-up

Machine learning models present multivariate statistical associations between our measured features. Thus, after identifying relationships in the antibody levels between the four disease severity groups in the previous PCA and PLSDA analyses, we compared the antibody levels between two severity groups against 22 PfEMP1 domains upregulated in severe malaria, 10 domains upregulated in uncomplicated malaria, 2 *P. falciparum* control proteins and one tetanus toxin control protein. We compared antibody levels between children with cerebral malaria and uncomplicated malaria at hospital presentation and during follow-up. We also determined the change in antibody levels between plasma collected at presentation and during follow-up in children with cerebral malaria and uncomplicated malaria. For all comparisons, antibody levels against all the PfEMP1 domains in the community controls and the Melbourne negative controls (malaria naïve) were significantly lower than children with either type of malaria, with the exception that antibody levels against tetanus toxin were higher in Melbourne negative controls (statistics for these comparisons have not been provided).

6.3.7.1 Antibody levels against individual PfEMP1 domains upregulated in severe malaria

We compared antibody levels in children with cerebral malaria to children with uncomplicated malaria against 22 PfEMP1 domains, transcripts of which were upregulated in severe malaria.

Antibody levels at hospital presentation (acute) against individual PfEMP1 domains upregulated in severe malaria

At hospital presentation, we found antibody levels to be significantly higher in children with cerebral malaria than children with uncomplicated malaria against 6 out of 22 domains upregulated in severe malaria (Figure 6.9, Table 6.6). For the other 16 domains, the antibody levels were either similar between the two severity groups or were higher in cerebral acute group, without these differences being significant (Figure 6.9, Table 6.6).

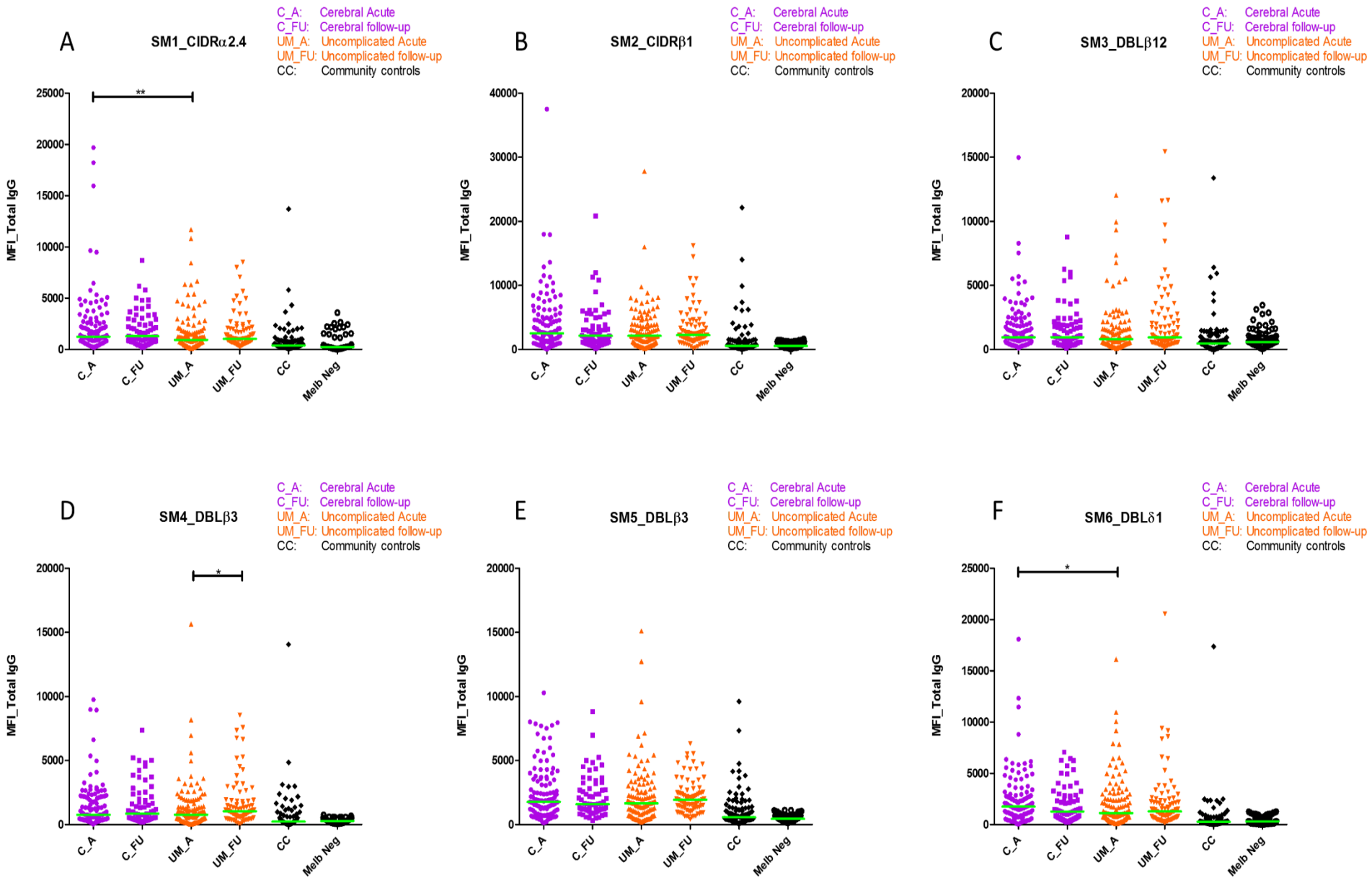
Antibody levels during follow-up against individual PfEMP1 domains upregulated in severe malaria

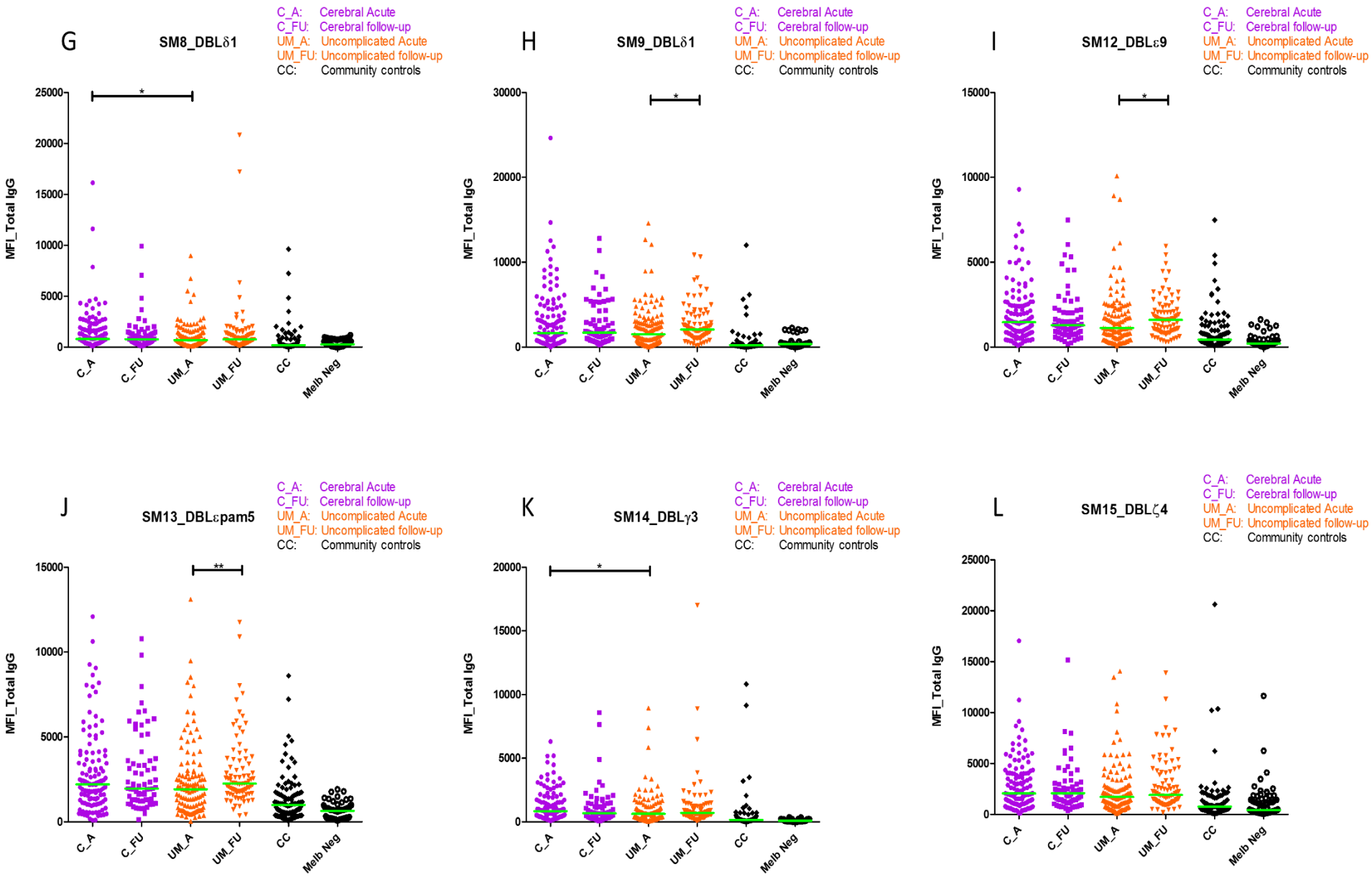
During the follow-up period, antibody levels against the 22 PfEMP1 domains whose transcripts were upregulated in severe malaria were compared between children with

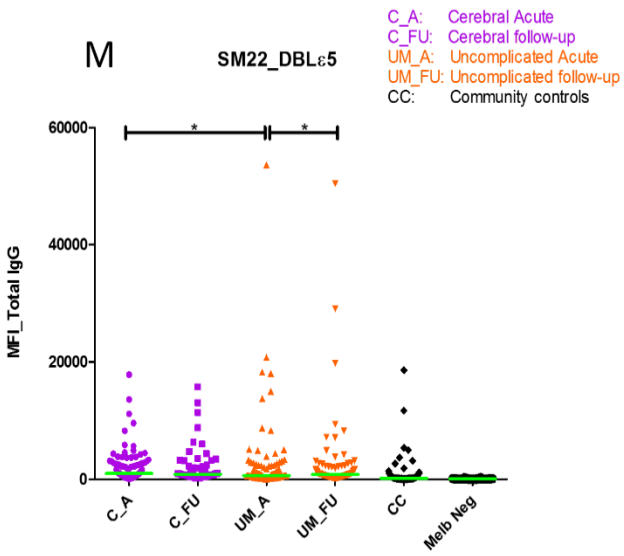
cerebral malaria and uncomplicated malaria. No significant differences in antibody levels to any of the PfEMP1 domains were observed (Figure 6.9).

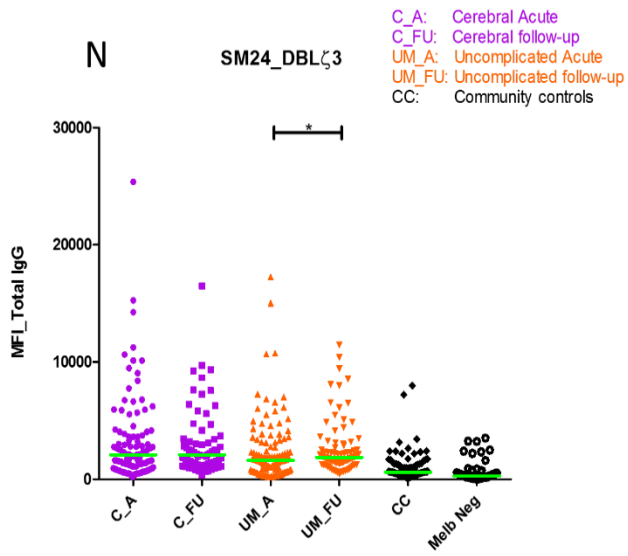
Change in antibody levels from presentation to follow-up against individual PfEMP1 domains upregulated in severe malaria

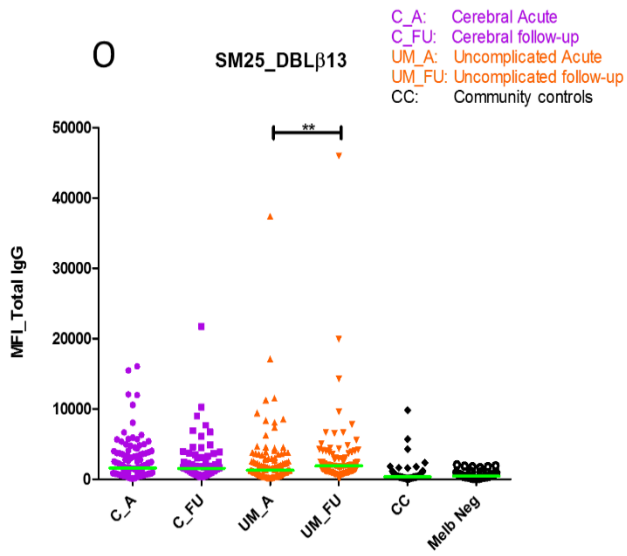
Antibody levels increased from presentation to follow-up in children with uncomplicated malaria. These differences were significant for 11 out of the 22 severe malaria domains tested (Figure 6.9, Table 6.6). In children with cerebral malaria, antibody levels were not significantly different between presentation and follow-up for all 22 PfEMP1 domains (Figure 6.9).

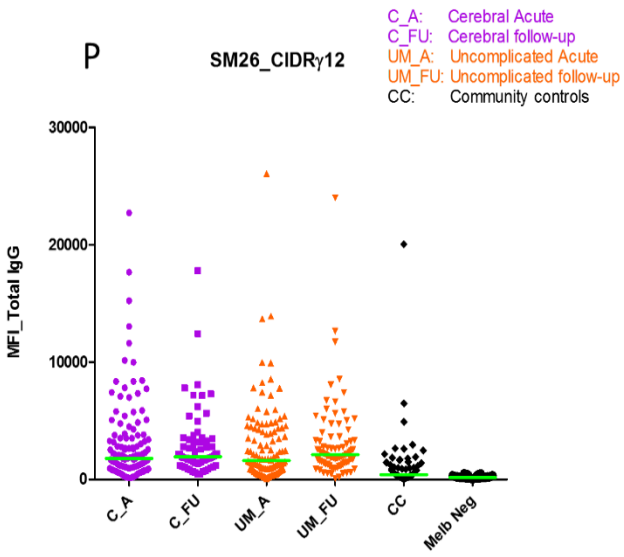

A
SM1_CIDRα2.4
B
SM2_CIDRβ1
C
SM3_DBLβ12
D
SM4_DBLβ3
E
SM5_DBLβ3
F
SM6_DBLδ1
C_A: Cerebral Acute
C_FU: Cerebral follow-up
UM_A: Uncomplicated Acute
UM_FU: Uncomplicated follow-up
CC: Community controls
MFI_Total IgG
C_A
C_FU
UM_A
UM_FU
CC
Melb Neg
**
*

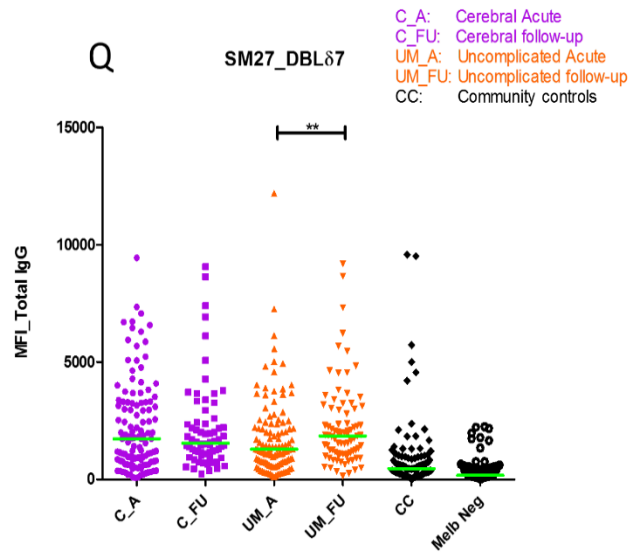

G
SM8_DBLδ1
H
SM9_DBLδ1
I
SM12_DBLε9
J
SM13_DBLεpam5
K
SM14_DBLγ3
L
SM15_DBLζ4
C_A: Cerebral Acute
C_FU: Cerebral follow-up
UM_A: Uncomplicated Acute
UM_FU: Uncomplicated follow-up
CC: Community controls
MFI_Total IgG
C_A
C_FU
UM_A
UM_FU
CC
Melb Neg

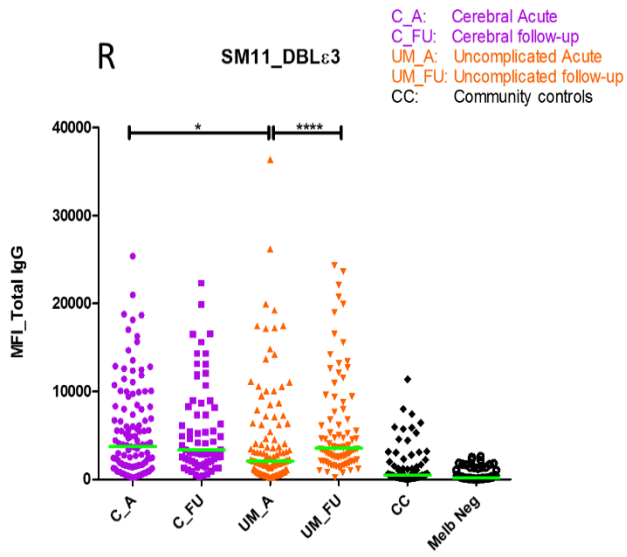

M
SM22_DBLε5
C_A: Cerebral Acute
C_FU: Cerebral follow-up
UM_A: Uncomplicated Acute
UM_FU: Uncomplicated follow-up
CC: Community controls
MFI_Total IgG
60000
40000
20000
0
*
*
C_A
C_FU
UM_A
UM_FU
CC
Melb Neg


N
SM24_DBLζ3
C_A: Cerebral Acute
C_FU: Cerebral follow-up
UM_A: Uncomplicated Acute
UM_FU: Uncomplicated follow-up
CC: Community controls
MFI_Total IgG
30000
20000
10000
0
*
C_A
C_FU
UM_A
UM_FU
CC
Melb Neg


O
SM25_DBLβ13
C_A: Cerebral Acute
C_FU: Cerebral follow-up
UM_A: Uncomplicated Acute
UM_FU: Uncomplicated follow-up
CC: Community controls
MFI_Total IgG
50000
40000
30000
20000
10000
0
**
C_A
C_FU
UM_A
UM_FU
CC
Melb Neg


P
SM26_CIDRγ12
C_A: Cerebral Acute
C_FU: Cerebral follow-up
UM_A: Uncomplicated Acute
UM_FU: Uncomplicated follow-up
CC: Community controls
MFI_Total IgG
30000
20000
10000
0
C_A
C_FU
UM_A
UM_FU
CC
Melb Neg


Q
SM27_DBLδ7
C_A: Cerebral Acute
C_FU: Cerebral follow-up
UM_A: Uncomplicated Acute
UM_FU: Uncomplicated follow-up
CC: Community controls
MFI_Total IgG
15000
10000
5000
0
**
C_A
C_FU
UM_A
UM_FU
CC
Melb Neg


R
SM11_DBLε3
C_A: Cerebral Acute
C_FU: Cerebral follow-up
UM_A: Uncomplicated Acute
UM_FU: Uncomplicated follow-up
CC: Community controls
MFI_Total IgG
40000
30000
20000
10000
0
*
****
C_A
C_FU
UM_A
UM_FU
CC
Melb Neg

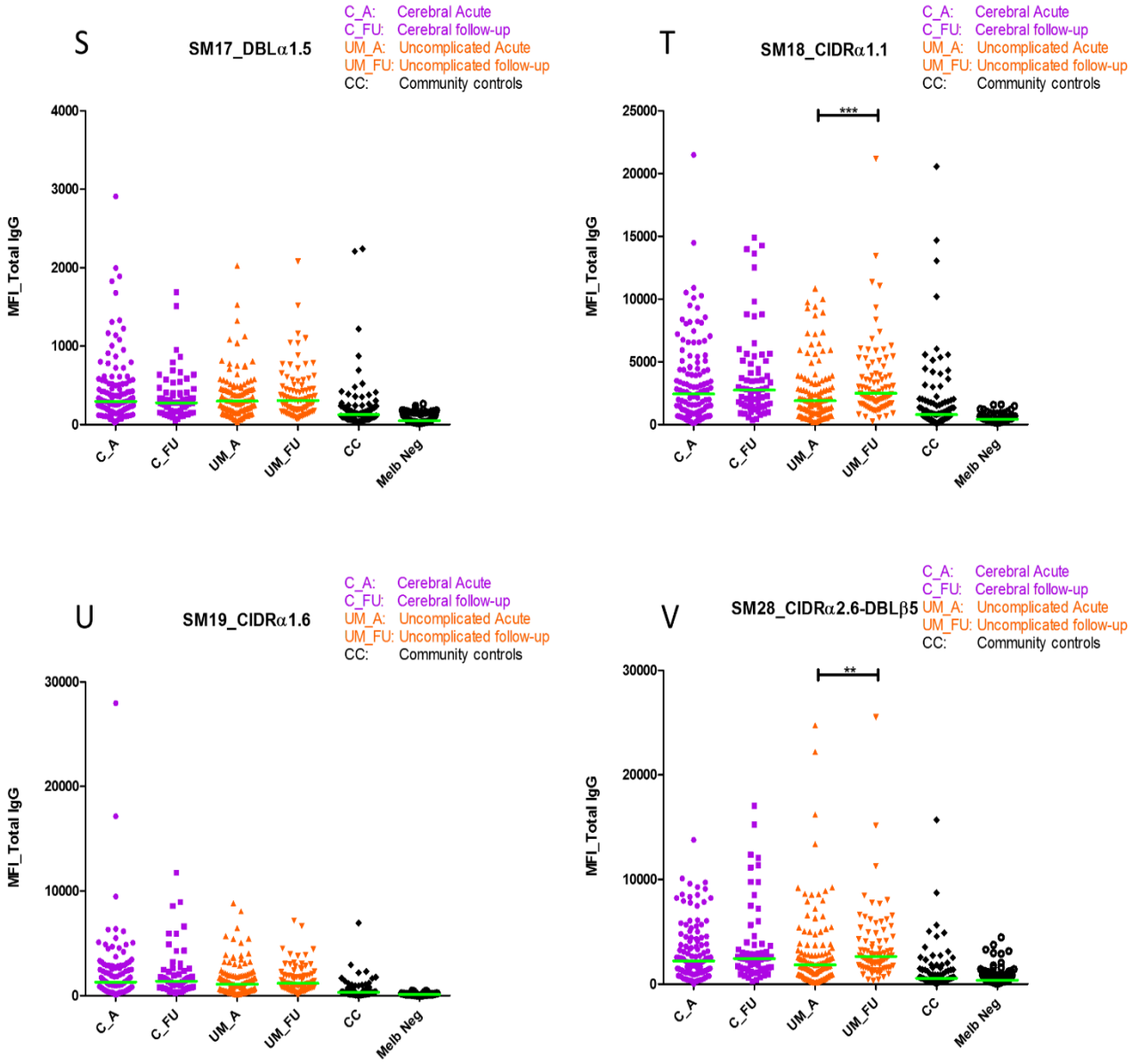

S
SM17_DBLα1.5
C_A: Cerebral Acute
C_FU: Cerebral follow-up
UM_A: Uncomplicated Acute
UM_FU: Uncomplicated follow-up
CC: Community controls
MFI_Total IgG
4000
3000
2000
1000
0
C_A
C_FU
UM_A
UM_FU
CC
Melb Neg
T
SM18_CIDRα1.1
***
25000
20000
15000
10000
5000
U
SM19_CIDRα1.6
30000
V
SM28_CIDRα2.6-DBLβ5
**

Figure 6.9 Antibody levels in Malawian children against PfEMP1 domains upregulated in severe malaria.

Using Wilcoxon Rank Sum test, individuals with cerebral malaria and uncomplicated malaria at acute and follow-up time points were compared. Y-axis represents the total IgG levels as median fluorescence intensity. Median antibody levels are presented as green horizontal bars for each disease severity group. Significant associations indicated by p-values: * < 0.05, ** < 0.01, *** <0.001, **** < 0.0001. Total number of participants in the cerebral acute malaria group, n = 119; cerebral malaria follow-up group, n = 68; uncomplicated acute malaria group, n= 118 and uncomplicated malaria follow-up group, n = 85. 77 antibody measurements from an average of 10 malaria naïve negative controls (from Melbourne) were also compared. A-V: Antibody levels were measured against PfEMP1 domains upregulated in patients with severe malaria. MFI, median fluorescence intensity; IgG, immunoglobulin G; SM, severe malaria; C_A, cerebral acute; C_FU, cerebral follow-up; UM_A, uncomplicated malaria acute; UM_FU, uncomplicated malaria follow-up; CC, community controls; Melb neg, malaria naïve negative controls from Melbourne.

6.3.7.2 Antibody levels against individual PfEMP1 domains upregulated in uncomplicated malaria

Next, we compared antibody levels against the 10 PfEMP1 domains, transcripts of which were upregulated in uncomplicated malaria cases, in children with cerebral malaria and uncomplicated malaria.

Antibody levels at hospital presentation (acute) against individual PfEMP1 domains upregulated in uncomplicated malaria

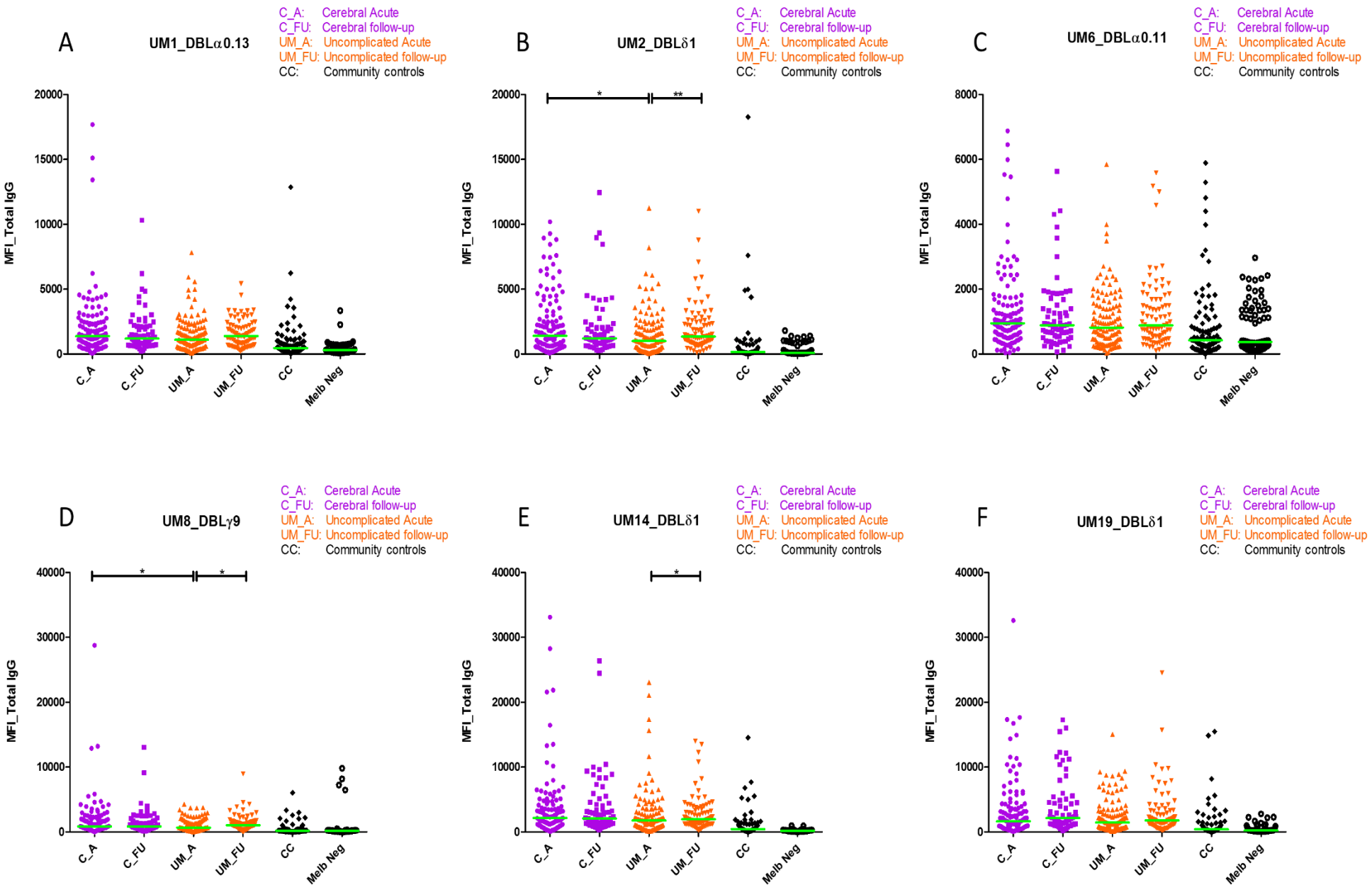
At presentation with disease, antibody levels were significantly higher in children with cerebral malaria as compared to those with uncomplicated malaria for only 2 out of 10 PfEMP1 domains, transcripts of which were upregulated in uncomplicated malaria cases. Differences in antibody levels were non-significant for the remaining 8 domains (Figure 6.10, Table 6.6).

Antibody levels during follow-up against individual PfEMP1 domains upregulated in uncomplicated malaria

Antibody levels were similar in children with cerebral malaria and uncomplicated malaria during the follow-up period for all 10 PfEMP1 domains upregulated in uncomplicated malaria (Figure 6.10).

Change in antibody levels from presentation to follow-up against individual PfEMP1 domains upregulated in uncomplicated malaria

In children with uncomplicated malaria, antibody levels increased from presentation to follow-up against 6 out of the 10 PfEMP1 domains whose transcripts were upregulated in uncomplicated malaria cases (Figure 6.10, Table 6.6). Antibody levels against all 10 domains remained unchanged over time in children with cerebral malaria (Figure 6.10).


A
UM1_DBLα0.13
B
UM2_DBLδ1
C
UM6_DBLα0.11
D
UM8_DBLγ9
E
UM14_DBLδ1
F
UM19_DBLδ1
C_A: Cerebral Acute
C_FU: Cerebral follow-up
UM_A: Uncomplicated Acute
UM_FU: Uncomplicated follow-up
CC: Community controls
MFI_Total IgG
C_A
C_FU
UM_A
UM_FU
CC
Melb Neg
*
**

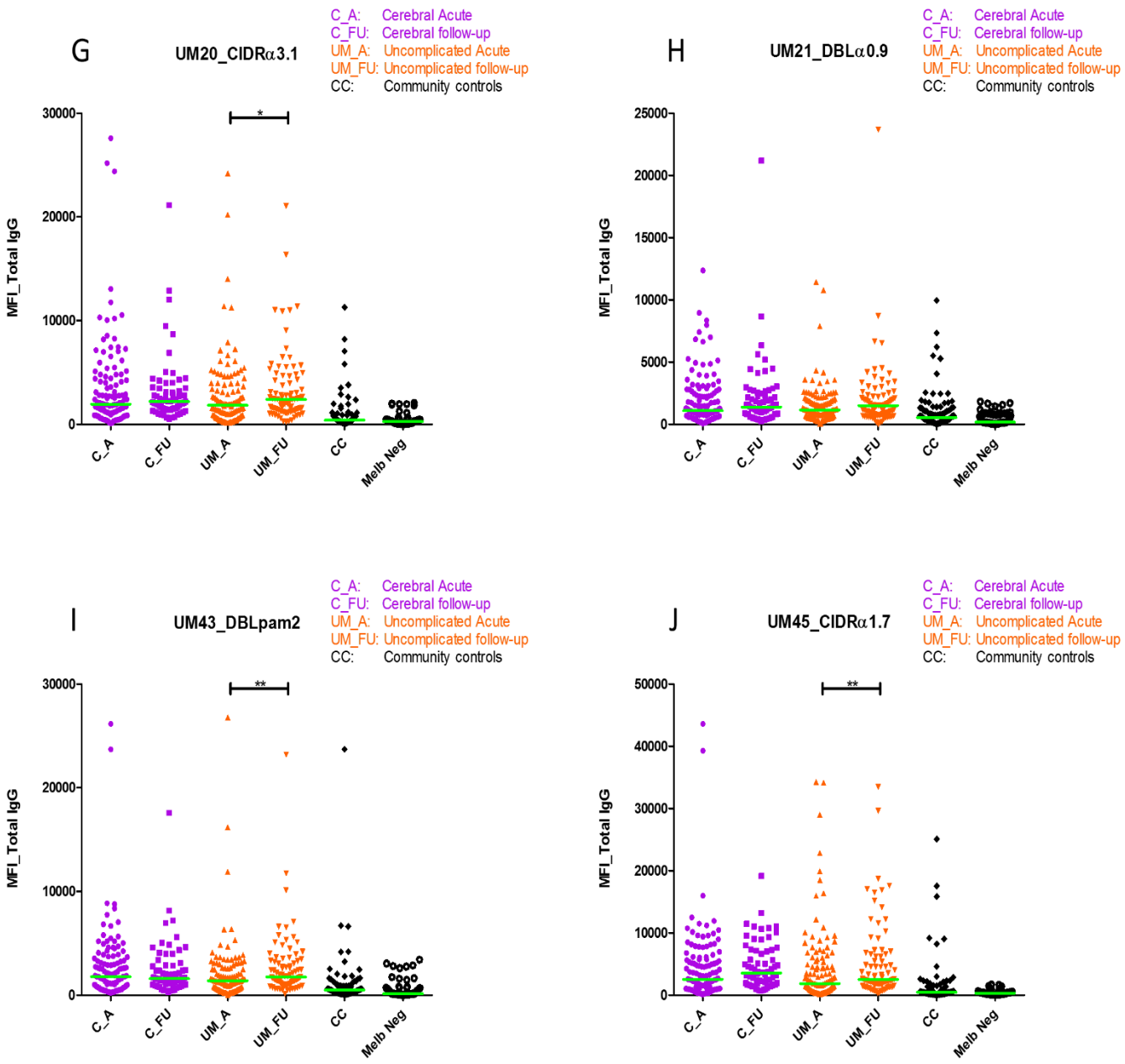

G
UM20_CIDRα3.1
C_A: Cerebral Acute
C_FU: Cerebral follow-up
UM_A: Uncomplicated Acute
UM_FU: Uncomplicated follow-up
CC: Community controls
MFI_Total IgG
30000
20000
10000
0
*
C_A
C_FU
UM_A
UM_FU
CC
Melb Neg
H
UM21_DBLα0.9
C_A: Cerebral Acute
C_FU: Cerebral follow-up
UM_A: Uncomplicated Acute
UM_FU: Uncomplicated follow-up
CC: Community controls
MFI_Total IgG
25000
20000
15000
10000
5000
0
C_A
C_FU
UM_A
UM_FU
CC
Melb Neg
I
UM43_DBLpam2
C_A: Cerebral Acute
C_FU: Cerebral follow-up
UM_A: Uncomplicated Acute
UM_FU: Uncomplicated follow-up
CC: Community controls
MFI_Total IgG
30000
20000
10000
0
**
C_A
C_FU
UM_A
UM_FU
CC
Melb Neg
J
UM45_CIDRα1.7
C_A: Cerebral Acute
C_FU: Cerebral follow-up
UM_A: Uncomplicated Acute
UM_FU: Uncomplicated follow-up
CC: Community controls
MFI_Total IgG
50000
40000
30000
20000
10000
0
**
C_A
C_FU
UM_A
UM_FU
CC
Melb Neg

Figure 6.10 Antibody levels in Malawian children against PfEMP1 domains upregulated in uncomplicated malaria.

Using Wilcoxon Rank Sum test, individuals with cerebral malaria and uncomplicated malaria at acute and follow-up time points were compared. Y-axis represents the total IgG levels as median fluorescence intensity. Median antibody levels are presented as green horizontal bars for each disease severity group. Significant associations indicated by p-values: * < 0.05, ** < 0.01, *** <0.001, **** < 0.0001. Total number of participants in the cerebral acute malaria group, n = 119; cerebral malaria follow-up group, n = 68; uncomplicated acute malaria group, n= 118 and uncomplicated malaria follow-up group, n = 85. 77 antibody measurements from an average of 10 malaria naïve negative controls (from Melbourne) were also compared. A-J: Antibody levels were measured against PfEMP1 domains upregulated in patients with uncomplicated malaria. MFI, median fluorescence intensity; IgG, immunoglobulin G; UM, uncomplicated malaria; C_A, cerebral acute; C_FU, cerebral follow-up; UM_A, uncomplicated malaria acute; UM_FU, uncomplicated malaria follow-up; CC, community controls; Melb neg, malaria naïve negative controls from Melbourne.

6.3.7.3 Antibody levels against control proteins

Antibody measurements against the three control proteins, AMA-1, CSP and tetanus toxin were made twice in Malawian children with malaria and are presented in figure 6.11. Antibody levels were found to be significantly higher in children with cerebral acute malaria as compared to children with uncomplicated acute malaria against AMA-1 ($p = 0.005$ and $p = 0.022$). At follow-up, there was weak evidence for antibodies against tetanus toxin to be higher in children with cerebral malaria compared to uncomplicated malaria ($p = 0.045$ and $p = 0.049$). No other significant differences in antibody levels were found on comparing different groups at presentation or during follow-up for any of the control proteins tested.

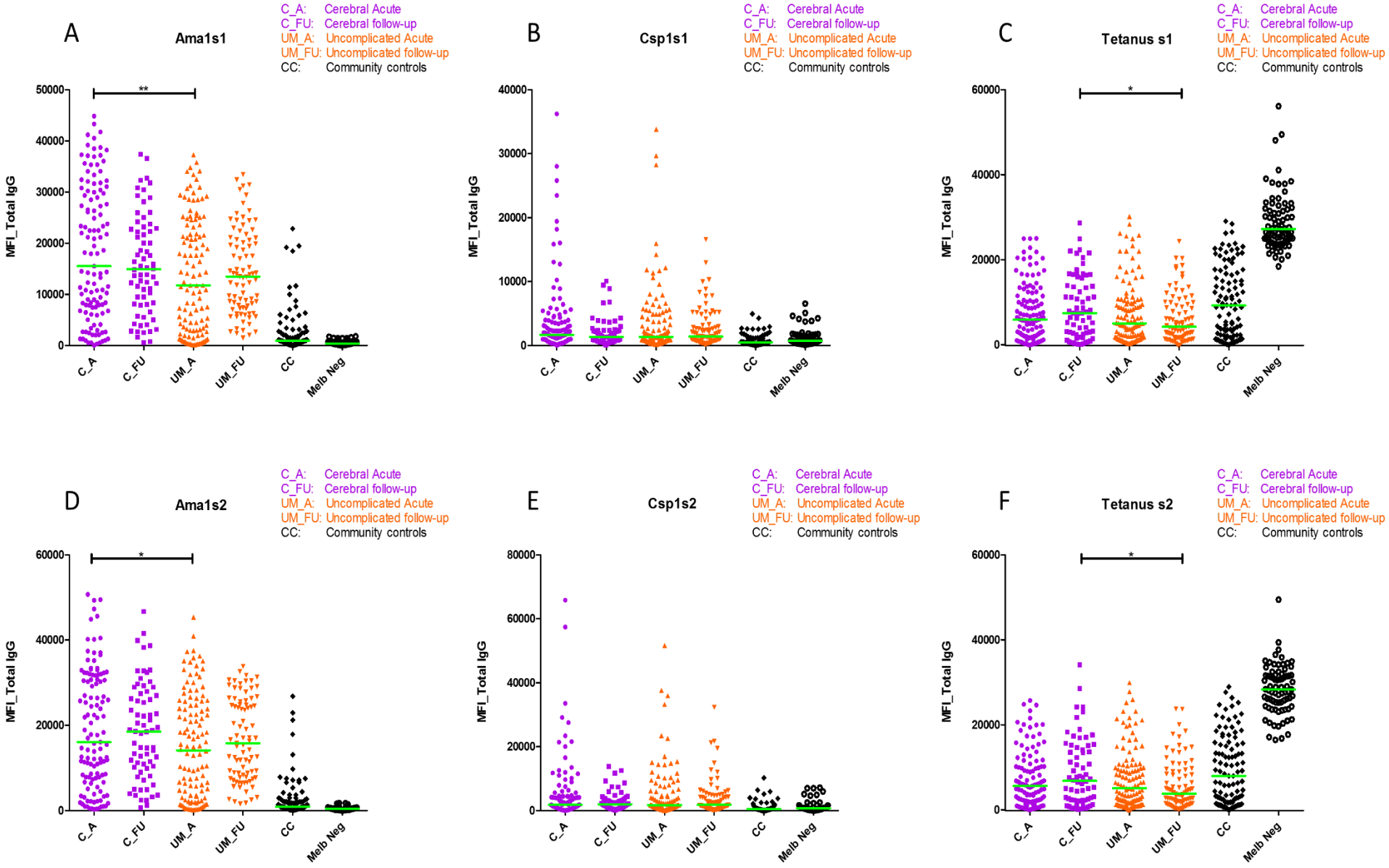

A
Ama1s1
B
Csp1s1
C
Tetanus s1
D
Ama1s2
E
Csp1s2
F
Tetanus s2
C_A: Cerebral Acute
C_FU: Cerebral follow-up
UM_A: Uncomplicated Acute
UM_FU: Uncomplicated follow-up
CC: Community controls
MFI_Total IgG
C_A
C_FU
UM_A
UM_FU
CC
Melb Neg
**
*

Figure 6.11 Antibody levels in Malawian children against control proteins.

Using Wilcoxon Rank Sum test, individuals with cerebral malaria and uncomplicated malaria at acute and follow-up time points were compared. Y-axis represents the total IgG levels as median fluorescence intensity. Median antibody levels are presented as green horizontal bars for each disease severity group. Significant associations indicated by p-values: * < 0.05, ** < 0.01, *** <0.001, **** < 0.0001. Total number of participants in the cerebral acute malaria group, n = 119; cerebral malaria follow-up group, n = 68; uncomplicated acute malaria group, n= 118 and uncomplicated malaria follow-up group, n = 85. 77 antibody measurements from an average of 10 malaria naïve negative controls (from Melbourne) were also compared. A-F: Antibody levels were measured against three control proteins (and were tested twice). MFI, median fluorescence intensity; IgG, immunoglobulin G; C_A, cerebral acute; C_FU, cerebral follow-up; UM_A, uncomplicated malaria acute; UM_FU, uncomplicated malaria follow-up; CC, community controls; Melb neg, malaria naïve negative controls from Melbourne.

Table 6.6 Differences in antibody levels between Malawian children with cerebral malaria and uncomplicated malaria at presentation and change in antibody levels from presentation to follow-up in children with uncomplicated malaria against PfEMP1 domains.

Sequence name	Domain name	Domain cassette	*Var* group	Binding phenotype	C_A vs UM_A	UM_A vs UM _FU
					P-value[a]	P-value[a]
SM1	CIDRα2.4.s.1		B	CD36	**0.008**	0.131
SM2	CIDRβ1.s.1	Non-DC4	B	Rosetting	0.113	0.296
SM3	DBLβ12.s.1	DC8	B/A	gC1qR	0.224	0.067
SM4	DBLβ3.s.1	Non-DC4	A	ICAM-1	0.457	**0.015**
SM5	DBLβ3.s.2	DC4 or non-DC4	A	ICAM-1	0.195	0.099
SM6	DBLδ1.s.1		B		**0.043**	0.483
SM8	DBLδ1.s.3		B		**0.019**	0.259
SM9	DBLδ1.s.4	DC8	B		0.077	**0.022**
SM11	DBLε3.s.1	DC11	B		**0.019**	**<0.0001**
SM12	DBLε9.s.1		B		0.086	**0.039**
SM13	DBLεpam5.s.1	VAR2	E		0.096	**0.003**
SM14	DBLγ3.s.1	DC9	B		**0.011**	0.117
SM15	DBLζ4.s.1	DC9	B		0.059	0.054
SM17	DBLα1.5		A	Rosetting	0.271	0.400

SM18	CIDRα1.1	DC8	B/A	EPCR	0.087	**0.001**
SM19	CIDRα1.6	DC4	A	EPCR	0.093	0.176
SM22	DBLε5	VAR1	A		**0.031**	**0.043**
SM24	DBLζ3		B/A		0.053	**0.019**
SM25	DBLβ13		B		0.193	**0.007**
SM26	CIDRγ12		B		0.539	0.061
SM27	DBLδ7		A		0.115	**0.008**
SM28	CIDRα2.6-DBLβ5		B		0.178	**0.002**
UM1	DBLα0.13.ns.1		B		0.064	0.111
UM2	DBLδ1.ns.2		B		**0.019**	**0.005**
UM6	DBLα0.11.ns.1		B		0.113	0.217
UM8	DBLγ9.ns.1		B/A		**0.027**	**0.017**
UM14	DBLδ1.ns.6		B		0.113	**0.043**
UM19	DBLδ1.ns.3		B		0.382	0.081
UM20	CIDRα3.1.ns.1		B/C	CD36	0.071	**0.019**
UM21	DBLα0.9.ns.1		B		0.263	0.062
UM43	DBLpam2.ns.1		E		0.073	**0.009**
UM45	CIDRα1.7.ns.1		A	EPCR	0.123	**0.009**

[a] the two groups were compared using Wilcoxon Rank Sum test.

C_A, cerebral acute; UM_A uncomplicated acute; UM_FU, uncomplicated follow-up; SM, severe malaria; DC, domain cassette; UM, uncomplicated malaria.

6.4 Discussion

Using novel bioinformatic analyses, several PfEMP1 domains were found to be differentially expressed in cases of severe malaria or uncomplicated malaria in individuals from Papua, Indonesia [141]. For the current study, we selected 28 PfEMP1 domains which were upregulated in severe malaria and 16 domains upregulated in uncomplicated malaria to study as targets of antibody immunity in malaria exposed individuals from Papua, Indonesia and children from Malawi. We were able to successfully clone and express 22 SM domains, 10 UM domains and 2 *P. falciparum* control proteins (table 6.4 and 6.5). Reasons for unsuccessful cloning include primers not binding to our template and thus unsuccessful PCR amplification or mutations introduced during cloning.

6.4.1 Protein expression and multiplex assay

We used the wheat germ cell-free protein synthesis system (WGCFS) to express HIS-tagged PfEMP1 domains cloned in *E. coli* expression vectors. Cell-based protein expression systems, have been widely used for expression of recombinant proteins in the past. However, these systems often lead to expression of incorrectly folded or insoluble proteins [317]. The major hindrances in expressing *falciparum* proteins using a cell-based system include the parasites' A/T rich genome and presence of repeated amino-acid motifs [90, 340, 341]. The eukaryotic, WGCFS can overcome these obstacles and result in production of high amounts of proteins as compared to other eukaryotic cell-free systems [316]. Further, this system is particularly useful for production of soluble, correctly folded, multi-domain proteins such as PfEMP1 [317]. Thus, this system allows us to study antibody profiles in malaria exposed individuals against recombinant PfEMP1 domains with conformations similar to those of native proteins. Future validation of the expressed proteins is required to confirm our current findings.

We measured total IgG levels against the PfEMP1 domains successfully cloned using the WGCFS. These proteins included domains whose transcripts were upregulated in severe malaria or uncomplicated malaria and *falciparum* control proteins. Antibody levels were measured against these domains in individuals with severe or uncomplicated malaria from Papua at presentation. These individuals formed the population that was also used to generate samples used to determine PfEMP1 domain transcripts upregulated in severe or uncomplicated disease. Similar measurements of total IgG to the PfEMP1 domains were made in Malawian children with cerebral or uncomplicated malaria at presentation and four weeks later (follow-up) as well as in age-matched healthy community controls. A high through-put multiplex assay was used for these measurements, which has potential to measure humoral responses against up to 500 antigens simultaneously [318]. In the

current study, antibody responses against 16 PfEMP1 domains and 3 control proteins were quantified per assay. One of the limitations of this assay is that we cannot differentiate cross-reactive antibodies targeting similar domains (with similar sequences) from specific antibody responses.

6.4.2 Machine learning analyses

We used machine learning tools, including supervised and unsupervised machine learning models, to identify relationships in antibody responses to PfEMP1 domains among the different disease severity groups tested and to assess if differences in antibody responses differed according to disease severity, or between presentation and convalescence. This approach is useful for analysing large datasets, hypothesis-testing, defining areas of our measures which can be further analysed using other tests or models and providing insights on antibody features associated with a clinical or functional response [336].

In the Papuan population, antibody responses in individuals with uncomplicated malaria clustered somewhat separately from those with severe malaria and most of the differences observed between groups were driven by 7 PfEMP1 domains (figure 6.1). This separation was supported by a relatively high leave one out cross validation score and low error scores for the model. The 7 PfEMP1 domains most associated with variation in antibody responses by severity included UM2-DBLδ1, transcripts of which were upregulated in uncomplicated malaria, and SM19-CIDRα1.6, SM17-DBLα1.5, SM15-DBLζ4, SM4-DBLβ3, SM1-CIDRα2.4 and SM18-CIDRα1.1, transcripts of which were upregulated in severe malaria. SM18-CIDRα1.1 domain was associated with antibody responses in only a few individuals with severe malaria in comparison to all other individuals with severe malaria who clustered quite far away from it. Out of these 7 domains, DBLζ4 and CIDRα2.4 have not been previously linked with severe malaria or antibody responses and thus are novel findings, while findings for CIDRα1s and DBLβ3 are consistent with previous studies.

In the Malawian population, antibody responses in children with cerebral or uncomplicated malaria at presentation and follow-up did not show distinct separation (figure 6.6). Further cluster analysis comparing only the cerebral acute and uncomplicated acute groups revealed that antibody levels in the two groups clustered somewhat distinctly (figure 6.7). Similar cluster analysis comparing antibody responses in children with uncomplicated malaria at presentation and follow-up revealed a slight separation (figure 6.8). However, evidence of clustering for both the models was weak based on the low leave one out cross validation score and combined variance scores and

high error scores. Although the patient populations seemed to cluster somewhat and the antibody response to all proteins seemed to be greater in certain patient groups, the proteins themselves did not segregate into clusters defined by varying degrees of reactivity with patient groups. Due to time constraints, feature selection, PLSDA model (as done for the Papuan population) could not be applied to the Malawian population.

Analysis of large datasets using machine learning provides us with multivariate statistical associations for our measured features [336]. Based on observations from the present antibody patterns, it is possible that the highly variable antibody responses in certain individuals may skew the antibody patterns presented by the PCA and PLSDA. To complement the findings from machine learning, to confirm if the differences are statistically significant or not and to identify associations between antibody responses and PfEMP1 domains known to be biologically relevant, but not detected by machine learning, identifying differences in antibody levels against individual PfEMP1 domains in patients with different disease severities using traditional statistical analyses is essential. In the following paragraphs, we have first discussed the overall trend in differences in antibody levels against the domains in the two populations, followed by differences in antibody levels against individual domains or a group of domains (based on similar binding phenotypes or DCs or previous associations with severe malaria) in both the study populations in parallel. Further analyses using quantitative mathematical modelling as well as future experiments confirming the biological importance of our findings (that is, function/role of these antibodies in severe or uncomplicated malaria) and validation of the present results using other study cohorts are required [336].

6.4.3 Overall trend in antibody responses against the different domains in the two study populations

AMA-1 (expressed on merozoites) and CSP (expressed on sporozoites) were selected as control antigens, antibody to which may give some indication of malaria exposure. The two control proteins were synthesized using the same system (WGCFS) used to express our domains of interest. Levels of antibody against AMA-1 and CSP did not differ significantly between severe and uncomplicated malaria in the Papuan population, consistent with overall similar previous exposure to malaria in the two groups (although different individuals showed markedly different levels of antibody) (figure 6.5). Because all the participants recruited for this study presented at the healthcare facility in Timika, Papua, they could have been from the neighbouring areas, thus explaining similar exposure to malaria. Since the transcripts for the SM-derived domains tested in this study were all upregulated in Papuan individuals with severe malaria, higher antibody levels

against most domains in the same population with uncomplicated malaria (compared to severe malaria) point towards a protective function for these antibodies (figure 6.2). Out of all the PfEMP1 domains tested, 7 domains were identified as contributors of maximum variation in antibody responses by severity. Thus, we can infer that UM2-DBLδ1, SM19-CIDRα1.6, SM17-DBLα1.5, SM15-DBLζ4, SM4-DBLβ3, SM1-CIDRα2.4 and SM18-CIDRα1.1 are the best candidate PfEMP1 domains, antibodies to which may protect from severe disease. This finding is novel for DBLζ4 and CIDRα2.4 domains.

On the other hand, Malawian children with cerebral acute malaria had higher antibody levels compared to children with uncomplicated acute malaria against AMA-1 protein (figure 6.11). This could indicate differences in exposure to malaria in the cerebral malaria group and uncomplicated malaria group before presentation. Although all children presented to a hospital in Blantyre, Malawi, we currently do not have data on which areas these children came from or were referred from. The differences in exposure could also account for the cerebral group having higher antibody levels than the uncomplicated malaria group against most PfEMP1 domains tested. Our results are in line with previous studies demonstrating that children with cerebral malaria have higher antibody levels than children with uncomplicated malaria against PfEMP1 antigens in Benin [230] or non-PfEMP1 antigens (AMA-1, MSP-1 and 2) in Malawi [342]. Children with uncomplicated malaria showed an increase in antibody levels from presentation to follow-up against majority of the domains and the antibody responses in these children at acute time point were lower than those with cerebral malaria. This suggests that the increase in antibody levels in uncomplicated malaria is related to the exposure during infection to similar domains or cross-reactive epitopes [343]. One other explanation for the trends in antibody levels observed in the children could be the time point at which these children presented to the hospital with either cerebral or uncomplicated malaria. Children with uncomplicated malaria could have presented to the hospital earlier in the course of their infection than the cerebral cases and thus continued to develop antibodies through to follow-up, whereas cerebral malaria cases could have already reached the maximal antibody levels by the time of hospital presentation. However, we lacked information on the duration of infection (which is hard to measure in the field) or duration of illness (which is variable in different individuals) which together with information on the time point the plasma samples were collected in these children could to some extent help corroborate the above hypothesis.

The young children in our study from Malawi were tested for antibody levels against PfEMP1 domains whose transcripts were upregulated in individuals from Papua, Indonesia. This could result in low antibody immunity due to low previous exposure to

domains or a lack of antibody immunity due to the origin of the PfEMP1 domains in these children. We found that the children did recognise PfEMP1 domains expressed by parasites circulating in Papua, with antibody levels against certain PfEMP1 domains being higher at presentation in children with cerebral malaria compared to uncomplicated malaria; and antibody levels increasing from presentation to follow-up in children with uncomplicated malaria for some PfEMP1 domains (figures 6.9 and 6.10). Several studies in the past have demonstrated that plasma antibodies from individuals from one region could recognise VSAs from a different region [78, 213]. The differences in antibody levels against PfEMP1 domains observed in the children could be indicative of the parasite strains that these children are commonly, sometimes or rarely exposed to. It is important to note that out of the 7 domains most associated with differential antibody responses (by severity) in the adults, only some domains resulted in a differential antibody response in the children. Although the antibody responses against some domains were up in cerebral malaria cases, multiple infections with the same domain types may be required to elicit protective antibody responses in children [214]. Further analyses determining the seroprevalence of antibodies in these children will help us better understand the role of specific PfEMP1 domains in cerebral infections.

Thus, exposure to malaria is an important aspect of antibody immunity studies and differentiating antibody responses as markers of previous exposure from functional/protective responses is always challenging [241, 344]. In our study we included participants from Papua, Indonesia, who were mostly adults, and children from Malawi. Malaria transmission in Papua is unstable [345], while Malawi is a high transmission area [30]. Acquisition of anti-malarial antibodies is age-dependent [190, 225, 346, 347] and the antibody repertoire against PfEMP1 domain types increases with repeated exposure to them [190, 218]. These reasons could explain the different trends in antibody responses in adults and children in our study and the differences in the domains recognised. In the Papuan individuals, the antibody repertoire could be particularly well developed against the current panel of PfEMP1 domains tested as these were also the proteins expressed by locally circulating parasites.

The overall trend in antibody responses observed in both populations suggest that both SM-derived and UM-derived domains were targets of antibodies in both study populations.

In adults, antibodies against UM and SM- derived domains could imply that the individuals were exposed to these domains over the years and hence have developed

antibodies against a broad range of PfEMP1 domains [161]. In the current study, the adults with uncomplicated malaria showed higher antibody levels compared to adults with severe malaria against 15 out of 22 SM domains and 7 out of 10 UM domains (figures 6.2-6.4). These SM and UM -derived domains belonged to either group A, B or B/A *var* types.

In our study, antibody responses in children with cerebral malaria (compared to uncomplicated malaria) were significantly higher for 6 out of 22 PfEMP1 domains whose transcripts were upregulated in severe malaria and 2 out of 10 domains upregulated uncomplicated malaria (figures 6.9 and 6.10). These SM and UM -derived domains belonged to either group A, B or B/A *var* types. Children with uncomplicated malaria showed boosting of antibodies from presentation to follow-up against 11 out of the 22 SM domains and 6 out of the 10 UM domains (figures 6.9 and 6.10). Antibody boosting was seen for domains belonging to groups A, B, B/A, B/C and E *var* types. Because the uncomplicated cases were exposed to both SM and UM-derived proteins, expression of these domains cannot be restricted to cerebral malaria, although they might still cause cerebral malaria. Antibodies against domains which were upregulated in uncomplicated malaria also raises the possibility that antibodies to both SM and UM -derived PfEMP1 domains could have similar roles as markers of exposure or infection. Alternatively, one could suggest antibodies to be highly cross-reactive.

Previous studies from Africa have suggested a serologically more conserved subset of VSAs to be associated with severe malaria [213]. However, in the present study, we did not find a unique subset of PfEMP1 domains to be associated with antibody immunity or protection from severe malaria. Rather, a 'total' PfEMP1 antibody response to several different PfEMP1 domains seems to be associated with protection from severe malaria in Papuan adults, with CIDRα1.6, DBLα1.5, DBLζ4, DBLβ3 and CIDRα2.4 domains being identified as strong targets of protective immunity. In Malawian children, the antibody responses seem to be markers of exposure to PfEMP1 antigens (as supported by antibody levels to AMA-1 differing between cerebral and uncomplicated malaria) rather than markers of protective immunity.

6.4.4 Antibody responses against individual PfEMP1 domains in the two study populations

An important point to remember in this section of the discussion is that while some of these domains have been assigned a DC, the domains also occur outside of these DCs. Some of these domains have been discussed with reference to DCs in some cases, but

the domains are not a representative of the DC only. Details of the *var* transcription for these domains have been explained in [141].

DBLδ1

We measured antibody levels against several DBLδ1 domains, transcripts of which were upregulated in either severe or uncomplicated malaria in Papuan individuals. Although some of the SM-DBLδ1 transcript levels were not abundant in severe malaria cases, they were significantly different between the two severity groups [141]. DBLδ1 domain transcript was also abundantly expressed in one cerebral malaria patient from PNG [161]. Using machine learning approaches, antibody to one of these DBLδ1 domains was an important discriminator between adults with uncomplicated malaria and severe malaria in Papua: UM2-DBLδ1 (figure 6.2 A). Surprisingly, this specific DBLδ1 domain was upregulated in uncomplicated malaria cases from the same study population, that is its sequence was a part of a cluster of sequences which were expressed at significantly higher levels in uncomplicated patients than severe malaria patients [141]. This suggests that the stronger immune response to this UM2-DBLδ1 sequence in uncomplicated malaria could be because it is a rarer sequence restricted to expression in uncomplicated malaria, leading to uncomplicated malaria cases to have more antibodies to this protein. Amongst Malawian children as well, antibody levels against this domain were higher in the cerebral group compared to uncomplicated group at presentation and significantly boosted from presentation to follow-up for uncomplicated malaria (figure 6.10 B). This suggests association of this specific DBLδ1 domain with uncomplicated malaria and development of antibody responses against it in children with cerebral and uncomplicated malaria. Similar trends in antibody levels in the adults and children were observed for the other DBLδ1 domains, although not all differences were significant. Association of this domain with severe disease has not been previously reported and binding characteristics are unknown as well. DBLδ5 is the only DBLδ domain to be characterised and was shown to be a part of DC5 binding to PECAM-1 [137]. DBLδ5 [161] and DC5 have been previously associated with severe malaria [120, 141] and anti-DC5 antibodies have been linked to protection from febrile malaria [137, 220, 223, 240].

CIDRα1.1 and CIDRα1.6 domains

CIDRα1.1 (DC8, group B/A) and CIDRα1.6 (DC4, group A) are EPCR-binding domains [63]. Children with uncomplicated malaria had higher antibody recognition of PfEMP1s containing DC8 and DC13 (CIDRα1.4-1.7) compared to PfEMP1s lacking these DCs [163, 216]. Antibodies to the EPCR-binding CIDR domains were also shown to be acquired more quickly than other CIDR domains and early in life in malaria exposed

individuals from Tanzania [229]. Antibodies against CIDRα1-containing PfEMP1 were also shown to be more intense in convalescent sera from Malian children with cerebral malaria compared to acute sera [214].

Both CIDRα1.6 and CIDRα1.1 were part of the 7 domains that resulted in maximum variation in antibody levels by severity in Papuan individuals (figure 6.2 B, G). Our current results for CIDRα1.6 are consistent with all previous findings for the EPCR-binding CIDR domains. Given that antibody levels against CIDRα1.6 domain were higher in uncomplicated malaria cases, we could infer that antibodies against this severe malaria-associated domain could confer protection from severe malaria in Papuan adults. Surprisingly, antibody levels against CIDRα1.1 domain did not show significant differences between the two disease severity groups in this population. Overall, these results are in line with our results from chapter 4, where we found PNG children with severe malaria acquired antibodies against EPCR-binding CIDRα1 domain types compared to children with uncomplicated malaria during convalescence. However, lack of differences in antibodies to CIDRα1.1 domain in Papuan adults with severe and uncomplicated malaria is not in line with our results from chapter 5. In chapter 5, we found opsonic antibody levels against an EPCR-binding parasite IE line which contains CIDRα1.1 domain to be higher in PNG children with uncomplicated malaria than severe malaria at hospital presentation and during convalescence. Antibody levels against this CIDRα1.1-containing PfEMP1 type were also boosted from presentation to convalescence in children with severe malaria. This could suggest that in Papuan adults, even though CIDRα1.1 and CIDRα1.6 transcripts were upregulated in severe malaria cases [141], antibodies against CIDRα1.6 (DC4, group A) domain, but not CIDRα1.1 (DC8, group B/A) were associated with protection. This raises the possibility that these two domains may not be antigenically cross-reactive [135], even though previous structural studies on different CIDRα1 domains have shown these domains to retain their shape and binding potential despite large sequence diversities [128].

In contrast, Malawian children with uncomplicated malaria showed a boost in the antibody levels from presentation to follow-up against CIDRα1.1 and not CIDRα1.6 domain (figure 6.9 T, U). This could be attributed to previous exposure to CIDRα1.1 domain in children with uncomplicated malaria which were further boosted by current infection with the same PfEMP1 type. The antibody levels against both the CIDRα1 domains did not differ between children with cerebral malaria and uncomplicated malaria at either time points even though transcript levels of DCs containing these domains were shown to be upregulated in children with severe malaria syndromes like cerebral malaria in Tanzania [120] and Malawi [160]. Previous studies have suggested that severe malaria is

associated with expression of PfEMP1 types against which individuals have low levels of pre-existing antibodies [78]. This could explain the similar antibody levels in cerebral and uncomplicated malaria cases in our study population and can be further corroborated by *var* transcription studies in parasites infecting this population. Alternatively, one could also suggest that parasites causing the cerebral malaria infection may not be expressing these CIDRα1 domains or that by the time children with cerebral malaria presented to the hospital, they had already developed maximal antibody responses (and hence antibody levels were not boosted with time).

Our results suggest that adults from Papua and children from Malawi are exposed to EPCR-binding CIDR domains. Differences in the type of CIDRα1 domains leading to differential antibody responses in both populations suggest that antibodies targeting CIDRα1.1 and CIDRα1.6 may not be sufficiently cross-reactive [135]. Multiple exposures to multiple CIDRα1 subtypes may be required to elicit antibody immunity against these domains. Other factors or domains present in the PfEMP1 molecule (containing the CIDRα1 domains) may also contribute to the development of naturally acquired immunity. These factors could include, but are not limited to, age and geographical location of the populations.

DBLα1.5 and DBLε domains

In the present Papuan study, DBLα1.5 was found using machine learning to be one of the 7 domains resulting in most variation in antibody responses by severity, with antibody levels in the uncomplicated group being greater than the severe malaria group (figure 6.2 C). This domain has been previously associated with IgM-positive rosetting and induction of broadly cross-reactive antibodies against clinically rosetting parasites [127], but no direct link with severity has been established. In Malawian children, antibody levels were similar across the two severity groups at both time points, suggesting parasites causing the current infection were not expressing this domain (figure 6.9 S).

Against DBLε domains, transcripts of which were upregulated in severe malaria cases [138, 141], the antibody levels were higher in Papuan individuals with uncomplicated malaria as compared to severe malaria. However, these differences were not significant. These ε domains include SM11-DBLε3, SM12-DBLε9, SM13-DBLεpam5 (pregnancy associated malaria) and SM22-DBLε5 (figures 6.3 O, G, H, J (Papua) and 6.9 R, I, J, M (Malawi)). Few studies have highlighted the role of specific DBLε domains in malaria severity. DC6, which contains a DBLε4 domain (C-terminal PfEMP1 domains) usually occur along with NTS-PfEMP1 types binding EPCR or CD36 or associated with rosetting [138]. DBLε domains were shown to bind to serum factors like IgM or α2-macroglobulin in

some studies [139, 140] or associated with IgM-positive rosetting [127]. DBLε3 and DBLε11 domains can belong to DC11 [123, 141] or can be found outside of specific DCs. DBLε3 was also one of the most highly expressed/upregulated domain in Papuan adults with severe malaria [141]. Our results suggest that although antibodies against DBLε domains are elicited, the antibodies may not be strongly associated protection from severe malaria in Papuan adults.

In contrast, Malawian children with uncomplicated malaria showed a significant boost in antibody responses against these four DBLε domains from presentation to follow-up. Further, antibody levels against SM11-DBLε3 and SM22-DBLε5 were significantly higher in children with cerebral malaria as compared to children with uncomplicated malaria at presentation. In Tanzania, DBL5ε domain was shown to be one of the first DBL targets to be recognised by plasma antibodies. The same study further showed that around 50% of Tanzanian children (aged 2-3) had antibodies that recognised the DBL5ε domain [220]. Thus, these results suggest that the Malawian children may have been exposed to DBLε3 and DBLε5 and developed differential antibody responses against them.

Taken together results from Papuan individuals and Malawian children suggest that adults and children are exposed differently to DBLε and DBLα1.5 domains, both of which have been previously implicated in IgM-positive rosetting.

DBLζ4 and DBLγ3

DBLζ5 (DC6) and DBLζ2 (DC11) have been previously characterised [123, 138, 141] but can also occur outside the specific DCs. However, binding phenotypes for DBLζ have not been fully determined. It has been suggested that ζ domains bind to IgM and α2 macroglobulin [139, 140] or are associated with IgM-positive rosetting [127].

DBLζ4 on the other hand was shown to belong to DC9 (binding characteristic unknown), with transcript levels being higher in Tanzanian children with uncomplicated malaria than severe malarial anaemia [120]. In the Papuan population, DBLζ4 was one of the most abundantly transcribed domains in the severe malaria group [141]. Until now, antibody immunity studies on these domains have been lacking. In the current study as well, the domain was one of the seven domains most associated with antibody responses in uncomplicated malaria in Papua, with antibody levels being higher than the severe malaria group (figure 6.2 D), suggesting a protective role for these anti-DBLζ4 antibodies. In children, antibodies against DBLζ4 did not differ significantly between any of the severity groups tested (figure 6.9 L), once again highlighting the differences in exposure to this domain in these two populations. It is important to note that expression of this

domain (appendix III E) was observed as two prominent bands (of sizes close to the approximate size of DBLζ4) on the SDS gel image. These could be truncated forms of the same protein. We cannot be sure which form (band) of the protein was the target of these antibodies. Future studies validating these proteins and confirming their binding phenotype (if possible) are required.

Along with DBLζ4, DBLγ3 is another domain belonging to DC9. Expression of both these domain types has been implicated in severe malaria in only one previous study [141]. Among the Papuan adults, antibody levels were higher in uncomplicated malaria compared to severe malaria against this domain (figure 6.3 I), while in Malawian children, at presentation those with cerebral malaria had higher antibody levels than those with uncomplicated malaria (figure 6.9 K). These results suggest that DBLγ3 may be an important domain as a correlate of protective immunity (in adults) or exposure (in children).

CIDRα2.4

CIDRα2.4 was also one of the 7 domains most associated with antibody responses in uncomplicated malaria patients from Papua. Previous studies have reported CIDRα2-6 domains to bind CD36 [129, 130] and CD36 binding PfEMP1s have been associated with asymptomatic malaria [136, 148, 149]. In our entire panel, this was the only CD36 biding domain to be associated with severe malaria, eliciting antibody responses in children and adults. Antibody levels were higher in uncomplicated malaria cases in adults (figure 6.2 F), while in children, antibody levels at presentation were significantly higher in cerebral malaria than uncomplicated malaria (figure 6.9A). Our results suggest that antibodies to CIDRα2.4 may be associated with protection from severe malaria in adults and exposure in children. This is consistent with one previous study, where CIDRα2.4 domain transcript was abundantly expressed in one child with cerebral malaria in PNG [161]. CIDRα2.4 domain could be a part of a PfEMP1 associated with severe malaria through adhesion of other domains, with epitopes of CIDRα2.4 domain being important targets of antibody immunity to severe malaria. Future studies identifying association of CIDRα2.4 with other PfEMP1 types and identifying antibody responses against this domain in other study populations could corroborate our findings here.

DBLβ3

DBLβ3 domain belonging to DC4 or DC13, both of which bind to ICAM-1 [125, 133], were shown to be associated with severe malaria [135]. This domain can also occur outside of either of these DCs [131, 136]. Transcript levels of DBLβ3 were shown to be higher in

Tanzanian children with severe malarial anaemia and cerebral malaria compared to children with uncomplicated malaria [120]. In the Papuan population, although non-DC4-DBLβ3 transcripts were not abundantly expressed in severe cases, they were differentially transcribed in severe and uncomplicated malaria [141]. In our analysis, machine learning identified the SM4-DBLβ3 domain that does not belong to DC4 (and which may or may not belong to DC13 or may not be part of any DC) as one of the 7 domains most associated with antibody responses in uncomplicated malaria and not the SM5-DBLβ3 domain that may or may not belong to DC4. However, antibody responses against both SM4 and SM5- DBLβ3 domains were higher in uncomplicated malaria compared to severe malaria (figure 6.2 E, figure 6.3 C). In children, antibody levels against SM4-DBLβ3 domain that is not part of DC4 were boosted from presentation to follow-up in uncomplicated malaria only, while the antibody levels against the SM5-DBLβ3 domain did not differ significantly between any of the severity groups (figure 6.9 D, E).

Our results suggest DBLβ3 domain not belonging to DC4 may be associated with uncomplicated malaria in the children. On the other hand, in Papuan adults, antibodies against DBLβ3 domains not belonging to DC4 may be associated with protection from severe malaria. These results are in line with studies showing anti-DBLβ3 (DC13) antibodies to be associated with protection from clinical malaria in PNG [134].

DBLβ12

DBLβ12 belongs to DC8, binds gC1qR and has been shown to be upregulated in severe malaria [123]. In Papuan individuals with severe malaria as well, transcript levels for these domains were upregulated [141]. The same study further suggested that DBLβ12 and DBLβ3 could be targets of cross-reactive antibodies. In the adults, antibody levels against DBLβ12 domain were higher in uncomplicated malaria rather than severe malaria (figure 6.3 B). Future studies examining if antibodies to DBLβ12 and DBLβ3 are cross-reactive are required. In children from Malawi, antibody responses to DBLβ12 domain did not differ in either of the groups, suggesting that although antibodies are generated against DBLβ12, the domain does not result in differential antibody responses based on disease severity (figure 6.9 C).

CIDRβ1

In the Papuan study, although transcription of CIDRβ1 (non-DC4) was not very high in severe malaria cases, the transcript levels were highly conserved in a very restricted population of severe malaria cases [141]. In the same adult population, there was strong

evidence for antibody levels being significantly higher in uncomplicated malaria compared to severe malaria (figure 6.3A). Thus, antibodies against this domain could protect from severe malaria. In contrast, antibody levels against this domain were similar in children with cerebral or uncomplicated malaria in Malawi (figure 6.9 B), suggesting that although CIDRβ1 domains are recognised by antibodies, the domain does not result in variation in antibodies by severity. The binding phenotype for this domain has not been defined, but studies from Rowe and group [61] and Ghumra and group [127] have suggested that CIDRβ/γ/δ domains may be associated with rosetting.

Other domains upregulated in severe malaria

DBLζ3, DBLβ13, CIDRγ12, DBLδ7 and CIDRα2.6-DBLβ5 domains were also upregulated in severe malaria cases in Papua, of which the last combined domain came up in multiple levels of transcript analyses [141]. Surprisingly, antibody levels against the combined CIDRα2.6-DBLβ5 transcript did not differ significantly in the Papuan disease groups. For all other domains, the antibody levels were significantly higher in uncomplicated malaria compared to severe malaria suggesting involvement of these domains in severe malaria in Papua (figure 6.3 K-N, P). In Malawi, children with uncomplicated malaria showed an increase in antibody levels from presentation to follow-up against all these domains (except CIDRγ12 where the increase was non-significant) (figure 6.9 N-Q, V), suggesting previous or current exposure to these domains or presence of cross-reacting antibodies.

Other domains upregulated in uncomplicated malaria

DBLα0.13, DBLα0.11 and DBLα0.9 were some of the DBLα0-like domains upregulated in Papuan individuals with uncomplicated malaria. Transcripts for DBLα0-like domains were predominantly transcribed in African children having uncomplicated malaria and a high parasite burden [155]. In our study, antibody levels against these domains (except DBLα0.9) were significantly higher in Papuan individuals with uncomplicated malaria compared to severe malaria, suggesting these domains to be targets of immunity (figure 6.4 A, B, G). However, antibody levels against these domains did not differ significantly by disease severity in the Malawian group, suggesting these domains may not be dominant targets of antibody immunity in Malawian children (figure 6.10 A, C, H).

SM13-DBLεpam5 (upregulated in severe malaria cases) and UM43-DBLpam2 (upregulated in uncomplicated malaria cases) are two pregnancy associated domains that were examined for antibody responses. Adults showed no differences in antibody levels to these domains based on disease severity (figure 6.3 H, figure 6.4 H). Children with uncomplicated malaria showed a boost in antibody levels to these domains from

presentation to follow-up (figures 6.9 J and 6.10 I). The Malawian children in the current cohort have a median age of around 4 years. Thus, these antibodies are unlikely to be maternally transferred given that maternal antibodies are quite short-lived, with an acute decline in antibody levels observed in children between the ages of 6 months and 1 year [217]. Antibodies against the pregnancy associated DBL domains in the Malawian children could be driven by maternal antibodies in children aged less than 1 year. Further analysis of the antibody responses against these domains after stratifying the children by age can help us confirm our hypothesis.

For all the other domains upregulated in uncomplicated malaria cases (figure 6.4 and 6.10), serologically, we saw similar antibody levels between the severity groups or a similar trend in antibody responses to these domains as seen above in both the study populations tested: in adults, antibody levels were higher in uncomplicated malaria than severe malaria; in children antibody levels were boosted from presentation to follow-up in uncomplicated malaria and/or higher in cerebral acute compared to uncomplicated acute malaria. However, not all differences were significant. These results suggest previous or current exposure to these domains or presence of cross-reactive antibodies.

On comparing antibody responses in children with cerebral malaria, we did not observe differences from presentation to follow-up against any of the PfEMP1 domains tested. We also did not observe differences in antibody levels on comparing children with cerebral and uncomplicated malaria during follow-up. Antibody responses observed in these groups could be due to exposure to these domains at the time of current infection (presentation) and maintenance of antibody levels during follow-up. These responses could also be due to previous exposure to these domains and lack of boosting in the current infection due to absence of expression of such PfEMP1 types.

Other domain cassettes

As previously discussed, it is important to note that some of the domains described as a part of a DC can also occur outside the DC or in other DCs or need not necessarily be associated with any DC. We measured differences in antibody levels against multiple domains belonging to the same DC in children from Malawi and adults from Papua. For example, we measured antibody levels against DBLβ12 (binds gC1qR) and CIDRα1.1 (binds EPCR), both of which belong to DC8. In adults, we found differences in antibody levels against DBLβ12 domain and not CIDRα1.1, while in the children, differences in antibody levels were observed against CIDRα1.1 and not DBLβ12 domain. A recent study

[214] suggested that the PfEMP1 domains associated with cytoadhesion may not be the dominant target of naturally acquired immunity. Together with the dual binding properties attributed to DC8-containing PfEMP1 [123], one can suggest either of the domains in the PfEMP1 molecule carrying the DC8 to be the dominant immune target. The differences in the domains recognised by antibodies from adults and children could be due to differences in age and exposure to malaria or maturity of the immune system [347], differences in geography and transmission or the interplay between cytoadhesion of the parasite, avoiding immune recognition and presence of pre-existing antibodies [78]. Similarly, we found multiple domains of DC9 (DBLγ3 and DBLζ4) to be associated with differences in antibody responses in adults and children. Adults showed differences in antibody levels against DBLγ3 and DBLζ4, but in children with cerebral malaria at presentation, antibody levels were higher only against DBLγ3 compared to children with uncomplicated malaria. Adhesion phenotypes for both these domains are currently unknown. Association of DC9 domains with severe malaria and antibody immunity is novel as no previous studies have found associations between the two.

CIDRβ1, DBLβ3 and CIDRα1.6, the other domains can be found in DC4 and elsewhere (that is, other DC or not part of any DC). Most of these domains have been previously associated with severe malaria or rosetting [120, 127, 135]. Although antibodies to DC4 or individual domains belonging to DC4 have not been directly linked with protection from severe malaria, some studies have highlighted the role of anti-DC4 antibodies in malaria [133, 134]. In the Papuan population, even though transcript levels for DC4 domains were highly upregulated, these three domains may be found within or outside DC4 or not part of a DC at all [141]. In the current study, while the CIDRβ1 and DBLβ3 domains tested do not belong to DC4, the CIDRα1.6 domain tested belongs to DC4. Using the same Papuan population, we found that the antibody levels against these 3 domains were higher in individuals with uncomplicated malaria compared to severe malaria. Using machine analysis, DBLβ3 and CIDRα1.6 domains were identified as part of the seven domains that showed most variation in antibody responses by severity, suggesting that antibodies against these domains could be protective. In Malawian children, we did not observe significant differences in antibody levels to CIDRβ1, DBLβ3 or CIDRα1.6 domains based on disease severity (except DBLβ3, where antibody levels were boosted from presentation to follow-up in uncomplicated malaria). This could suggest that parasites expressing PfEMP1s containing these domains may not be responsible for the current cerebral infection or uncomplicated infection.

6.4.5 Summary of discussion and conclusion

Thus, this study helped us gain insights on antibody immunity in two different study populations against a large panel of PfEMP1 domains. Transcripts for these domains were upregulated in clinical malaria cases as opposed to previous *var* transcript studies which were based on laboratory-based parasite isolates. Knowledge of antibody immunity in the Asia-Pacific region is limited compared to African regions, and our study further increased our understanding of antibody responses in individuals with severe malaria from Papua, Indonesia. Similarly, antibody profiles in children with severe malaria and severe malaria syndromes such as cerebral malaria are also limited. Cerebral malaria is one of the major causes of malaria mortality and antibody studies against various PfEMP1 domains such as ours, can contribute towards improving our understanding of pathogenesis of severe malaria, identifying which antigens are associated with protection from severe malaria and potential protective strategies. However, PfEMP1 is a complex, multi-domain protein and we could not identify a unique, conserved set of PfEMP1 domains to be associated with antibody immunity or protection in our study. We did identify several novel PfEMP1 domains as targets of antibody immunity and confirmed some of the previously known associations between antibodies and PfEMP1 domains.

Due to time constraints, we were limited in the type of analyses we could do to assess differences in antibody levels against various PfEMP1 domains between adults and children. The Papuan adults with severe malaria in our population had mixed etiology for severe malaria, such as cerebral malaria, jaundice, hyperparasitaemia. The Malawian children on the other hand were characterised as only having cerebral malaria. However, due to limited numbers of Papuan individuals with the different severe syndromes, we could not identify differences in antibody responses based on differences in the severe symptoms presented. Further detailed analyses to understand differences in antibody levels based on age (adults versus children) and geographical regions (Asia-Pacific versus Africa) are required. Further experiments determining the IgG subclasses involved, functional role for these antibodies and *var* transcription of parasites infecting the Malawian study group can help us better understand antibody immunity against PfEMP1.

In summary, we can say that antibody responses to various PfEMP1 domains vary based on geographical region and age of the population, even though the PfEMP1 domain transcripts associated with severe malaria were shown to be broadly similar across different regions by previous studies. These antibody differences could also be attributed to differences in exposure to malaria. EPCR-binding CIDR domains (CIDRα1.1 and 1.6)

were significantly associated with severe malaria and antibody immunity as widely shown in previous studies and in chapter 4 and 5 of this thesis. We also found associations in antibody responses by disease severity to PfEMP1 domains such as DBLβ3, DBLα1.5 and DCs such as DC8 and DC4 which were previously implicated in severe malaria. The novel domains identified as immune targets in the present study included DBLζ4 and CIDRα2.4, antibodies to which differed by disease severity in the Papuan adults and/or Malawian children. These antibody responses could be markers of previous or current infections, cross-reactive antibodies due to previous or current infections and boosting over follow-up or indicate a protective role for antibody against these domains. A summary of the antibody responses against all the PfEMP1 domains tested in both populations has been provided in table 6.7.

Thus, with respect to our hypotheses for this thesis (chapter 2), we found that 1) the antibody profile for children differed from the antibody profile for adults. 2) In adults, the antibody levels were lower in individuals with severe malaria compared to uncomplicated malaria. 3) results for children severe malaria was not in complete agreement with our hypothesis. The antibody levels were not low at presentation (compared to uncomplicated malaria) and were not boosted over the follow-up period. This could be partly attributed to differences in the exposure to these domains between children with severe malaria compared to uncomplicated malaria.

Our studies have also demonstrated that although transcripts of certain PfEMP1 domains may be abundant in individuals with severe malaria, they may not necessarily be associated with variation in antibody immunity by severity. For example, DBLε3 transcripts were highly upregulated in Papuan individuals with severe malaria. But antibody responses against this domain did not differ by disease severity. Differentiating PfEMP1 domains associated with high transcription in severe malaria cases from domains inducing antibody responses is essential for future vaccine studies. The success of developing a PfEMP1 based vaccine lies in identifying domains associated with protection, understanding the acquisition of antibody responses against PfEMP1 proteins, their boosting and maintenance as well as identifying the functional mechanisms underlying anti-PfEMP1 immunity [170]. In our study, we have observed that antibodies to a unique, conserved set of PfEMP1 domains cannot be associated with antibody immunity in severe infections. Further, acquisition of antibodies to PfEMP1 domains is different in children and adults, although antigens from different geographical regions may be conserved. Thus, identifying multiple PfEMP1 domains most associated with antibody immunity (including boosting and maintenance of antibody levels) in severe malaria cases

and identifying antibodies capable of inducing protective functions is the way forward for PfEMP1 based vaccines.

Table 6.7 Summary of antibody responses against all PfEMP1 domains tested in Papuan and Malawian population

Sequence name	Domain name	Domain cassette	*Var* group	Binding phenotype	Papua	Malawi	
					SM < UM	C_A > UM_A	UM_A < UM _FU
SM1	CIDRα2.4.s.1		B	CD36	*	**	
SM2	CIDRβ1.s.1	Non-DC4	B	Rosetting	**		
SM3	DBLβ12.s.1	DC8	B/A	gC1qR	*		
SM4	DBLβ3.s.1	Non-DC4	A	ICAM-1	*		*
SM5	DBLβ3.s.2	DC4 or non-DC4	A	ICAM-1	*		
SM6	DBLδ1.s.1		B		*	*	
SM8	DBLδ1.s.3		B		*	*	
SM9	DBLδ1.s.4	DC8	B				*
SM11	DBLε3.s.1	DC11	B			*	****
SM12	DBLε9.s.1		B				*
SM13	DBLεpam5.s.1	VAR2	E				**
SM14	DBLγ3.s.1	DC9	B		*	*	

SM15	DBLζ4.s.1	DC9	B		*		
SM17	DBLα1.5		A	Rosetting	*		
SM18	CIDRα1.1	DC8	B/A	EPCR			***
SM19	CIDRα1.6	DC4	A	EPCR	**		
SM22	DBLε5	VAR1	A			*	*
SM24	DBLζ3		B/A		*		*
SM25	DBLβ13		B		**		**
SM26	CIDRγ12		B		*		
SM27	DBLδ7		A		*		**
SM28	CIDRα2.6-DBLβ5		B				**
UM1	DBLα0.13.ns.1		B		*		
UM2	DBLδ1.ns.2		B		***	*	**
UM6	DBLα0.11.ns.1		B		*		
UM8	DBLγ9.ns.1		B/A		**	*	*
UM14	DBLδ1.ns.6		B		**		*

UM19	DBLδ1.ns.3	B			
UM20	CIDRα3.1.ns.1	B/C	CD36		*
UM21	DBLα0.9.ns.1	B			
UM43	DBLpam2.ns.1	E		*	**
UM45	CIDRα1.7.ns.1	A	EPCR	*	**

Comparisons were made between two disease severity groups using the Wilcoxon Rank Sum Test; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$

SM, severe malaria; UM, uncomplicated malaria; C_A, cerebral acute; UM_A, uncomplicated malaria acute, UM_FU, uncomplicated malaria follow-up; DC, domain cassette.

7 Chapter 7: Final discussion and conclusion

The aim of the current study was to investigate the relationship between different measures of antibody to PfEMP1 and severity and stage of malaria disease. While assessing antibody responses by stage of disease could provide information on the type of PfEMP1 causing an infection, antibody measures based on severity of disease could help us determine if the antigens studied are targets of malaria exposure or if they could have a potential protective role. PfEMP1 is an important immune target. Studying antibody immunity to surface antigens of the IE, in particular PfEMP1, is essential to understand the pathogenesis of severe malaria in malaria endemic regions. This in turn will advance the possible future development of a PfEMP1 based vaccine. Development of PfEMP1 based vaccines has so far been impeded by the antigenic diversity and variability of the protein. The successful development of a PfEMP1 based vaccine would first require identifying conserved sets of PfEMP1 domains capable of eliciting protective antibody responses, gaining a better understanding of acquisition of antibody responses against various PfEMP1 types or PfEMP1 domains or domain cassettes, boosting and maintenance of the antibody responses as well as identifying the underlying functional mechanisms of anti-PfEMP1 immunity [170]. Ideally, the vaccine should induce cell-mediated and antibody immune responses, work in both children and adults and work across different regions having different transmission intensities. It is also important to address if only a PfEMP1 vaccine would work or if we need to look into combination vaccines which would include immune responses to other VSAs presented on the IE.

For the current thesis, unique, high-throughput antibody measurement assays were employed to study anti-PfEMP1 antibody immunity across different study populations. The first aim was to determine if recombinant CIDR domains of PfEMP1 were targets of antibody immunity in PNG children with severe or uncomplicated malaria. A multiplex assay, comprising of beads coupled to 35 recombinant CIDR domains was used to measure antibody levels in plasma collected from these children at hospital presentation and in convalescence. Next, to determine if differences in functional antibodies to whole parasites expressing the native PfEMP1 molecule in the same study population as in aim 1, an opsonic phagocytosis assay was employed. This assay measured levels of opsonising antibodies against four PfEMP1 types expressed by four different parasite IE: an ICAM-1 binder (which also binds CD36), a CD36 binder, an EPCR binder and IE expressing a PfEMP1 associated with rosetting. The final aim was to express PfEMP1 domains -transcripts of which were shown to be upregulated in severe malaria clinical isolates from Papua- using the wheat germ cell free protein synthesis systems and

evaluate them as targets of antibody immunity. A multiplex assay comprising of beads coupled to 32 expressed, recombinant PfEMP1 domains was used to measure antibody levels in Papuan adults and Malawian children with severe or uncomplicated malaria.

7.1 Main findings

7.1.1 Recombinant CIDRα1 domains of PfEMP1 which bind EPCR are important targets of antibody immunity

The hypothesis behind the first aim was that antibodies against CIDR domains in children with malaria differ by disease severity and/or between hospital presentation and convalescence. In recent years, EPCR-binding PfEMP1 types or domains (CIDRα1) have been implicated in severe malaria at the transcript level in several studies [120, 138, 141, 159-161]. There have been antibody immunity studies against EPCR-binding PfEMP1 as well [125, 163, 229], but studies demonstrating a direct link with disease severity have been few. Further, majority of the antibody studies were based in African regions.

The findings relating to PfEMP1 antibody are novel, revealing associations between anti-CIDR antibody immunity and disease severity in young children from PNG. Of the three CIDR domain types tested, antibodies against recombinant CIDRα1 domains, which are known or predicted to bind EPCR, were higher in convalescent plasma from older children with severe malaria compared to children with uncomplicated malaria. Antibody levels against CIDRα1 domains which belong to either group A or group B/A (DC8) *var* types also increased from presentation to convalescence in children with severe malaria, but not uncomplicated malaria. These results are in line with a recent study of Malian children with cerebral malaria or severe anaemia, which showed intense antibody responses against PfEMP1s containing CIDRα1 domains in convalescent sera compared to acute sera. Such boosting was observed in children with severe disease only. In that study, serum reactivity was not directly measured against CIDRα1 domains, but against fragments of PfEMP1s containing the CIDRα1 domain [214]. The age-stratified responses observed in the current study could be due to maturation of the immune system [347] or anti-PfEMP1 immunity increasing with age [190, 225, 346] and gradually with repeated exposure to malaria infections [190, 218], as opposed to early studies demonstrating that in cases of non-cerebral severe malaria (which require hospitalization), immunity develops as early as the first or second infections [217].

Studies investigating the relationship between different measures of antibody to PfEMP1 domains and individual severe malaria syndromes presented are limited, with one study demonstrating anti-CIDR1α antibodies (VAR4 family) to be associated with a reduced risk

of developing severe malarial anaemia [346]. Several studies have linked the transcription of EPCR-binding PfEMP1 domains or domain types to severe malaria syndromes (as well as other severe malaria categories) such as cerebral malaria or severe malarial anaemia in Tanzania [159], and cerebral malaria in Benin [147], Malawi [160] and PNG [161]. In Kenyan children with severe malaria, impaired consciousness was associated with expression of group A-like *var* genes and absence of rosetting [150]. In the present study, antibodies against the EPCR-binding CIDR domains did not differ significantly between children with severe malaria who had severe anaemia or hyperlactataemia or impaired consciousness and children who had severe malaria but none of the above syndromes. Antibodies against the group B/A CIDR EPCR domains types were higher in convalescent plasma from children with impaired consciousness (BCS 0-4) compared to children with uncomplicated malaria. Together these results suggest that antibodies against CIDR EPCR domain types are acquired against all major severe malaria syndromes in PNG children following exposure, and do not differ substantially between different syndromes of severe disease.

The current study demonstrated the acquisition of antibodies against EPCR-binding CIDRα1 domains of PfEMP1 most prominently after severe malaria and the influence of age on immunity to severe disease in PNG. Strengths of the study also include measurement of antibodies against several CIDR domains of PfEMP1 and providing novel insights on the type of PfEMP1 associated with severe malaria in PNG.

Thus, these findings imply that parasites expressing the EPCR-binding PfEMP1 domains may be involved in the pathogenesis of severe malaria in PNG, and that these domains are targets of antibodies which in turn are acquired by children with severe malaria more than they are acquired by children with uncomplicated malaria.

7.1.2 Antibodies against CIDRα1-containing PfEMP1 molecules which bind to EPCR function partly through opsonic phagocytosis

Naturally acquired antibodies to surface antigens expressed on the IE can have varied functions such as inhibition of parasite adhesion to host receptors, inhibition of rosetting, or opsonic phagocytosis of the IE by monocytes (reviewed in [170, 241]). Based on this, the hypothesis for the second aim was that functional or opsonising antibodies directed against parasite IE expressing the native PfEMP1 molecules with different binding phenotypes differed by disease severity and stage.

Role of opsonic phagocytosis in placental malaria has been previously well studied and level of opsonising antibody has correlated with parasite clearance and reduction in the

risk of maternal anaemia [264]. Further, loss of opsonic phagocytosis due to HIV co-infections was suggested to result in an increased risk of pregnancy associated malaria [265, 266]. Opsonic phagocytosis as an immune function is less understood in severe childhood malaria. Using the same PNG study population as in aim 1, antibodies against PfEMP1 were shown to function partly by opsonic phagocytosis [323]. Using four PfEMP1 types, the current study was one of the first studies to find levels of opsonising antibodies against EPCR-binding PfEMP1 to differ by disease severity. Opsonic antibody levels against EPCR-binding PfEMP1 were higher in children with uncomplicated malaria compared to severe malaria, further suggesting that these antibodies could potentially protect from severe malaria. Antibodies against EPCR-binding PfEMP1 were higher in children with uncomplicated malaria compared to children with either severe malarial anaemia or impaired consciousness or hyperlactataemia. Naturally acquired immunity to severe malaria syndromes can be syndrome-specific or non-syndrome-specific [214]. The current study demonstrated that opsonising antibodies against the EPCR-binding PfEMP1 did not differ based on the syndrome presented and hence were non-syndrome specific. Thus, having antibodies to PfEMP1 variants causing severe malaria (without stratifying by severe syndromes) could also protect from individual severe malaria syndromes. This is an important finding as studies determining the role of functional antibodies against specific severe malaria syndromes have been limited. Severe anaemia, respiratory distress and impaired consciousness are the severe malaria syndromes that often overlap in African children with severe malaria, with impaired consciousness and respiratory distress being the strongest predictors of mortality [46]. Understanding the relationship between measures of antibody to PfEMP1 based on specific syndromes presented will aid in the development of protective strategies against severe malaria.

The EPCR-binding IE line used for the current aim comprises of DC8 which includes DBLγ6, DBLβ12, CIDRα1.1 and DBLα2 domains and belongs to group B/A *var* genes [120]. On the other hand, the recombinant CIDRα1 domains used in the previous aim include CIDRα1.1 and 1.8 (group B/A, DC8) and CIDRα1.4-1.7 (group A), all of which either bind to or are predicted to bind to EPCR [128]. Despite conflicting results for EPCR binding and distinction between recombinant domains binding EPCR versus IE carrying the domain of interest and binding EPCR [126, 128], consistent with the findings from aim 1 -where antibodies against recombinant CIDRα1 domains binding EPCR were measured- opsonising antibody levels against the EPCR-binding IE line (DC8, group B/A) were boosted from presentation to convalescence in aim 2. The results from aim 1 were not indicative of protection from severe malaria but showed variable acquisition of

antibodies against CIDR EPCR domain types by disease state. Thus, results from aim 2 were complementary to aim 1 results and in addition showed that antibodies against EPCR-binding PfEMP1 could protect from severe malaria. Studies similar to the present one, measuring antibody levels against IE lines containing other CIDRα1 domains such as CIDRα1.4 (group A) or CIDRα1.6 (group A, DC4) are required to understand if antibody immunity to PfEMP1s containing different CIDRα1 variants correlate with each other.

These results suggest that the CIDRα1 domains and DC8-containing PfEMP1 are important targets of antibody immunity against severe malaria, but further studies exploring the presence of other domains along with the CIDRα1s are required. The interplay between multiple domains on the same PfEMP1 molecule could explain the differences in the pattern of antibody immunity observed against PfEMP1 fragments/recombinant domains and IE expressing the native PfEMP1. Other VSA families could also be targets of antibody immunity and cannot be ignored even though PfEMP1 was shown to be the major VSA eliciting antibody immunity using knockdown laboratory IE isolates [190]. This is because, the non-PfEMP1 VSAs in these IE lines may or may not be similar to the ones observed in vivo [348].

For the rosetting IE, opsonising antibodies were higher in children with uncomplicated malaria compared to severe malaria during convalescence. These antibodies could be indicative of cross-reactive antibodies due to similarities in the exposed epitopes of group A PfEMP1 types [206, 329]. Opsonising antibodies against the CD36-binding PfEMP1 did not differ by disease severity and declined from presentation to convalescence in severe and uncomplicated malaria. The results suggest that these antibodies could be cross-reactive or that the *var* gene expression of some of the parasites causing both severe and uncomplicated malaria in these children could have been broadly similar to the CD36-binding IE line tested, but that they do not have a role in influencing the severity of clinical disease in this population. Opsonising antibodies against the ICAM-1-binding PfEMP1 (which also binds CD36) were boosted from presentation to convalescence in children with uncomplicated malaria, suggesting that parasites expressing these PfEMP1 variants could be causing uncomplicated malaria in PNG. This is in agreement with the type of ICAM-1 binder the IE line used in the present study is, that is, an ICAM-1/CD36 binder (and not ICAM-1/EPCR binder). Future studies using study cohorts such as the present one, to evaluate antibody levels against PfEMP1 belonging to DC4 or DC13 and dually binding ICAM-1 and EPCR, which have been implicated in severe malaria [135], are required to corroborate the current findings.

Thus, the current study found antibodies targeting EPCR-binding PfEMP1 to function partly through opsonic phagocytosis with potential to protect from severe malaria in young children from PNG. Such data for opsonising antibodies have been lacking for children from Asia-Pacific or African regions. Strengths of the study also include use of parasite IE which express the entire PfEMP1 molecule with different binding phenotypes as opposed to recombinant PfEMP1 domains. This provides a representation of PfEMP1 types which is closer to the native PfEMP1 molecules.

7.1.3 Novel PfEMP1 domains associated with differences in antibody immunity in Papuan adults and Malawian children

To gain a better understanding of antibody immunity to the multi-domain PfEMP1 protein, several DBL and CIDR domains, transcripts of which were upregulated in clinical isolates from Papuan individuals with severe or uncomplicated malaria were studied as targets of immunity in the final aim.

In the current study, machine learning approaches using supervised and unsupervised models such as PLSDA and PCA respectively helped analyse these large datasets, to define potentially protective antibody measures against specific PfEMP1 domains which can be explored further. These models demonstrated that differences in antibody levels by disease severity in the Papuan population were driven by 7 PfEMP1 domains: DBLδ1 (UM-derived domain), and CIDRα1.6, DBLα1.5, DBLζ4, DBLβ3, CIDRα2.4 and CIDRα1.1 (SM-derived domains). Since the transcripts for these 7 domains were upregulated in the same Papuan population, these domains could be the best candidate PfEMP1 domains, antibodies to which may protect from severe disease. This finding is novel for DBLζ4 and CIDRα2.4 domains. Analyses of the Malawian population showed that the antibody responses against the PfEMP1 domains clustered only somewhat distinctly by severity and the PfEMP1 proteins themselves did not segregate into clusters defined by varying degrees of reactivity with patient groups. Thus, these analyses resulted in insights into the relationships between antibody immunity against the antigenically variable PfEMP1 protein and severe malaria.

The Papuan adults with uncomplicated malaria showed higher antibody levels compared to adults with severe malaria against 15 out of 22 domains upregulated in severe malaria and 7 out of 10 domains upregulated in uncomplicated malaria cases. The domains eliciting differences in antibody levels by severity belonged to either group A, B or B/A *var* types. These results suggest that the individuals with severe malaria have lower antibody responses compared to individuals with uncomplicated malaria against a wide range of PfEMP1 domains (with restrictions, as group C *var* types were absent). Individuals with

uncomplicated malaria could have been exposed to these domains over the years and hence have developed antibodies against a broad range of PfEMP1 domains [161] with potential to protect from severe infections.

Antibody responses in Malawian children with cerebral malaria were significantly higher compared to children with uncomplicated malaria for 6 out of 22 PfEMP1 domains (whose transcripts were upregulated in severe malaria) and 2 out of 10 domains (upregulated in uncomplicated malaria). These domains belonged mainly to group B, A or B/A *var* types. Further, children with uncomplicated malaria showed boosting of antibodies with time against 11 out of the 22 SM-derived domains and 6 out of 10 UM-derived domains. Antibody boosting was seen for domains belonging to groups A, B, B/A, B/C and E *var* types.

Of all the PfEMP1 domains resulting in differences in antibody responses in Malawian children with malaria, some domains overlapped with the domains recognised as immune targets by Papuan individuals, while some domains did not overlap. For example, DBLε domains' transcripts were upregulated in Papuan individuals with severe malaria but did not result in differences in antibody levels by severity. However, the Malawian children with uncomplicated malaria showed strong evidence for antibody boosting against the DBLε domains, suggesting these domains to be early targets of immune responses and children with cerebral malaria already having antibodies against them to some extent. One could also suggest that the domains to which antibody responses were boosted in convalescence in uncomplicated malaria could be representative of the domains expressed by parasites causing the current infection.

Previous studies have linked uncomplicated malaria with the transcription of PfEMP1 types not associated with severe malaria or non-group A PfEMP1 types [120, 148, 149]. However, in the present study, antibodies from children and adults with uncomplicated malaria recognised PfEMP1 domains belonging to group A and non-group A PfEMP1 types.

Together, these results suggest that PfEMP1 domains upregulated in Papua are targets of immunity in Malawi as well, indicating a global conservation of PfEMP1 as immune targets [78, 213].

However, given the differences in the range of PfEMP1 domains recognized by antibodies in both the study populations (table 6.7), a unique, conserved set of PfEMP1 domains cannot be said to elicit antibody responses in individuals from different regions which in turn could protect from severe malaria. Previous studies have suggested a

conserved subset of PfEMP1s to be expressed by parasites causing severe malaria [151, 155, 349] and that this subset is better recognised by sera from semi-immune children compared to antigens expressed by parasites causing uncomplicated malaria [219, 350]. However, the current study did not find such evidences. Rather, a 'total' PfEMP1 antibody response to several PfEMP1 domains seems to be associated with protection from severe malaria in Papuan adults, with CIDRα1.6, DBLα1.5, DBLζ4, DBLβ3 and CIDRα2.4 domains being identified as strong targets of protective immunity. On the other hand, in young Malawian children with cerebral malaria, the antibody responses seem to be markers of exposure to PfEMP1 antigens (as supported by antibody levels to AMA-1 differing between cerebral and uncomplicated malaria) rather than markers of protective immunity.

If a particular antigen is responsible for the current severe infection, the antibody levels are likely to be boosted from hospital presentation to follow-up period [343]. Similar boosting was observed for the PNG children with severe malaria in aim 1 and 2 of the current study. Such boosting over four weeks was not observed in the Malawian children with cerebral malaria. This could suggest that parasites causing the cerebral malaria infection may not be expressing these domains or that by the time children with cerebral malaria presented to the hospital, they had already developed maximal antibody responses (and hence antibody levels were not boosted with time). Knowing the duration of infection (which is hard to measure in the field) or duration of illness (which is variable in different individuals) which together with information on the time point the plasma samples were collected in these children could to some extent help corroborate the above hypothesis. To determine if the children with uncomplicated malaria continue to be protected from future cerebral infections, measuring antibody levels in study cohorts where individuals are followed prospectively is required.

The current study identified several PfEMP1 domains, transcripts of which were upregulated in severe malaria clinical isolates from Papuan adults [141], as novel targets of antibody immunity. Previous studies measuring antibody levels against a range of PfEMP1 domains relied on PfEMP1 domains or fragments obtained from a parasite reference strain genome: 3D7 [214, 220, 351]. The novel domains identified as immune targets in the present study included DBLζ4, DBLδ1 and CIDRα2.4, antibodies to which differed by disease severity in the Papuan adults and/or Malawian children. Future studies identifying the binding phenotypes for these domains and determining if these domains are part of DCs or if the domains interact with other domains known to be associated with severe malaria are required.

Although previous studies have highlighted the role of anti-DC4 antibodies in malaria [133, 134], a direct link between anti-DC4 antibodies and disease severity has not been established. The current study was one of the first studies to find antibody levels against CIDRβ1, DBLβ3 and CIDRα1.6 to be higher in Papuan adults with uncomplicated malaria compared to severe malaria. It is important to note that while these three domains are found in DC4, they are also found in other DCs or not linked to any DC. In the current study, while the CIDRβ1 and DBLβ3 domains tested do not belong to DC4, the CIDRα1.6 domain tested belongs to DC4. Out of these three domains, DBLβ3 and CIDRα1.6 were identified by machine learning analysis to be most associated with variation in antibody responses by severity. These results suggest a protective role for antibodies targeting these two domains. In contrast, Malawian children did not show significant differences in antibody levels to any of these domains based on disease severity (except DBLβ3, where antibody levels were boosted from presentation to follow-up in uncomplicated malaria, suggesting that these children may have been exposed to these domains in the past and current infection with the same type may have resulted in antibody boosting with time). This could suggest that parasites expressing PfEMP1s containing these domains may not be responsible for the current cerebral infection or uncomplicated infection in Malawi. Analogous to the study in aim 2 using EPCR-binding parasite IE line (Var19) carrying DC8, future studies examining antibody levels against parasite IE expressing the entire PfEMP1 molecule carrying either CIDRβ1, DBLβ3 or CIDRα1.6 (having protein conformations closer to the native protein) will help us get a clearer picture of antibody immunity against these domains.

Antibodies to the EPCR-binding CIDRα1.6 domain were higher in Papuan adults with uncomplicated malaria compared to severe malaria, suggesting a protective role for antibodies against this domain. This is consistent with the findings from aim 1 and 2 as well. In Malawian children, only children with uncomplicated malaria showed antibody boosting from presentation to convalescence against the CIDRα1.1, EPCR-binding domain, suggesting previous exposure to this domain and/or boosting of antibodies over time due to current infection with PfEMP1s containing this domain. Antibodies against the CIDRα1.1 and 1.6 domains did not differ significantly between children with cerebral malaria and uncomplicated malaria even though transcripts for these domains were shown to be upregulated in cerebral malaria cases (and also other syndromes or severe malaria categories) in several studies [120, 147, 160, 161]. This could be explained by previous studies suggesting that severe malaria is associated with expression of PfEMP1 types against which individuals have low levels of pre-existing antibodies [78, 228]. Alternatively, it is possible that parasites causing cerebral malaria may not be expressing

CIDRα1.1 or CIDRα1.6 domains or that that by the time children with cerebral malaria presented to the hospital, they had already developed maximal antibody responses. Future *var* transcription studies in parasites infecting this population along with information on the duration of infection could help corroborate our hypothesis. However, measuring the duration of infection is practically difficult. Knowledge on duration of illness (although variable in different individuals) along with the timepoints the plasma samples were collected, could to some extent provide us with information on antibody acquisition patterns. Given the dual binding properties attributed to EPCR-binding PfEMP1 [123, 125] and results from current aim, one could suggest that other PfEMP1 domains present on the same PfEMP1 molecule as CIDRα1s could also be the targets of antibody immunity [141]. For example, a recent study by Chan and group [323] demonstrated that out of the four domains belonging to DC8 (DBLγ6, DBLβ12, CIDRα1.1 and DBLα2), DBLγ6 and DBLα2 were more important as targets of protective immunity. In the present study, transcripts of DBLγ6 were not upregulated in severe malaria cases in Papua and hence were not studied as immune targets. Transcripts of DBLα2 were upregulated in Papuan individuals with severe malaria, but the cloning of the domain was unsuccessful and due to time constraints could not be pursued further. Attempts to clone, express and examine DBLα2 as an immune target in the two populations are required.

In the current study two different panels of PfEMP1 proteins were coupled to beads and used to measure antibody levels in individuals with different malaria severities using multiplex assays. The first panel only comprised of different CIDR domains with different known or predicted binding phenotypes, while the second panel included DBL and CIDR domains with known or unknown binding phenotypes. Such broad multiplex assays involving antibody measurements against multiple recombinant proteins simultaneously are important to identify and narrow down PfEMP1 domains important for antibody acquisition, boosting and maintenance. Importance of such broad assays was also shown by several studies using PfEMP1 domains or fragments in multiplex assays or microarrays [214, 220, 229, 351]. This information will aid in the development of PfEMP1 based vaccines. Although protein expression systems used to produce recombinant proteins often result in generation of functional proteins with conformations similar to the native proteins, they may still expose epitopes that are generally not exposed to the immune system in vivo. Thus, once important PfEMP1 domains have been identified, assays measuring antibody levels against parasite IE expressing the entire PfEMP1 protein carrying our domains of interest are necessary (as done in aim 2 of this thesis). Such studies will provide a better understanding of the working of anti-PfEMP1 antibody immunity in vivo in conjunction with other VSAs on the IE, particularly as antibody titres

measured against recombinant proteins may temporally change in a different manner compared to opsonising antibody titres measured against IEs [352].

In the first aim, out of all the CIDR domains included in the panel, the CIDR domains binding EPCR were identified as important targets of antibody immunity in PNG. For the last aim, the protein panel included several DBL and CIDR domains. EPCR-binding CIDR domains were shown to be important targets of antibody immunity in Papuan adults and Malawian children as well. The pattern of antibody acquisition against PfEMP1 domains in each panel was different. These differences could be attributed to differences in age of the population, previous malaria exposure, current malaria exposure as well as the geographical location. Future correlation studies examining if antibody levels against one individual domain correlate with antibody levels against another domain which is either similar to the first domain (as in the CIDR panel) or belongs to the same DC as the first domain (as in the DBL and CIDR mixed panel) are required. These studies can also help identify targets of cross-reactive antibodies. 'Competition' antibody assays, where multiple antigens compete for binding to antibodies from one plasma sample could also potentially help identify which domain strongly correlates with antibody recognition/binding. For assays measuring antibody levels against the entire PfEMP1 molecule expressed by parasite IE, knocking out one domain of interest and generating a new 'knockout' parasite IE line (for example using CRISPR-Cas9 technology) could help us validate the importance of the knocked-out PfEMP1 domain in antibody immunity.

Strengths of the current study include the feasibility of cloning and expressing a large number of PfEMP1 domains from parasite isolates infecting clinical samples using WGCFS. The multiplex assay allowed us to measure antibody levels against a large panel of PfEMP1 domains using a high-throughput assay in big cohort studies. Machine learning approaches allowed us to analyse these big datasets thus generated. Overall, this study resulted in an improved understanding of antibody immunity to the multi-domain PfEMP1 protein.

Thus, overall the results suggest that the Papuan domains were recognised by antibodies from the Malawian study population and that parasites expressing similar PfEMP1 domains could be responsible for infections in both geographical regions. However, in the children, significant differences in antibody levels by disease severity were observed only against certain domains and were indicative of exposure rather than protection. The domains recognised by plasma from Malawian children were sometimes different from domains inducing differences in antibody responses by severity in adult plasma. These results suggest that acquisition of antibodies against PfEMP1 domains follows a different

pattern in children compared to adults or that responses differ by geography. Further analyses exploring differences in antibody levels to the different PfEMP1 domains in children versus adults are required. Future studies comparing antibody responses against a range of PfEMP1 domains in two study populations from different geographical settings as in the current study but having similar age groups (that is either only adults or only children) would help in understanding antibody acquisition and maintenance for individuals across different regions. Also, including participants with similar malaria exposure history but different disease severities could help us differentiate antibody responses that are markers of exposure from markers of immunity.

7.2 Overall limitations and future directions

The current study has some limitations. Studies determining antibody responses to defined antigens and differences in antibody levels by disease severity only provide an indirect indication of the parasite phenotypes that may be involved in the pathogenesis of severe or uncomplicated malaria. *Var* transcription studies for parasites infecting these populations could corroborate the current findings. Differentiating antibody responses as markers of exposure from antibody responses as markers of immunity was difficult. Information on the previous malaria exposure in all three study populations could have helped differentiate the type of antibody responses. While studying antibody responses to specific PfEMP1 domains, it is possible that within the single domain multiple antigenic sites/epitopes may be present. This may result in a differential or heterogeneous antibody response to the same domain. Thus, many more future studies are required to validate the findings from the current preliminary study as well as other such studies before PfEMP1- based vaccines can become a reality.

Limitations for the PNG study (aim 1 and 2) include small numbers of individuals with specific severe malaria syndromes which limited the type of analyses that could be done to understand antibody immunity in protection from severe syndromes. Having found EPCR-binding PfEMP1 domains to be important immune targets, future studies determining other roles for antibodies targeting the EPCR-binding PfEMP1 such as inhibition of CIDRα1-EPCR binding, dissecting the role of other VSAs in this immunity and cell-mediated immunity are required.

For the MAGPIX study (aim 3) further validation of the expressed proteins is required such as confirming the binding of the domains to known receptors (if the binding phenotype is known) or comparing the sequence of our cloned proteins to known databases/PfEMP1 domains using BLAST. This can also help us with the quality assessment of some of the expressed proteins which showed multiple bands in the gel

images (for example, appendix III A, E, K, of which DBLζ4 showed us interesting results). This quality assessment is important as these variations in the proteins may impact the epitopes exposed to antibodies. Another limitation for the MAGPIX multiplex assay is that cross-reactive antibodies targeting similar sequences or domains cannot be differentiated from specific antibody responses. We could not account for cross-reactivity in our study and future works assessing the cross-reactive nature of these antibodies are required.

In the Papuan and Malawian studies, differences in antibody responses to PfEMP1 domains based on age of the participants (adults versus children) as well as geographical locations (Papua versus Malawi) could not be fully explored due to time constraints. Further analyses investigating if the differences in antibody levels observed by disease severity (within and among the two populations) are driven by age, exposure, or geographical location are required. Comparing antibody levels between severe and uncomplicated malaria against the PfEMP1 domains after grouping the domains based on a particular 'type' as done in the first aim are also required. For example, grouping the EPCR binders or ICAM-1 binders or grouping all domains belonging to one DC. Similarly, machine learning approaches (as used for aim 3) applied to the CIDR panel in aim 1 could have helped identify specific CIDR domains most associated with antibody responses in severe malaria. Further *var* transcription studies for the parasites infecting the Malawian children are also required to understand the interplay between antibody immunity and transcription of PfEMP1 domains. Although antibodies were measured against a wide range of PfEMP1 domains in the Malawian population, it is possible that the PfEMP1 types causing cerebral malaria in Malawi were not part of the PfEMP1 panel upregulated in the Papuan population and studied as antibody targets. However, overall, the findings from aim 3 are consistent with previous studies on Malawian children with cerebral malaria with malarial retinopathy, where transcripts of EPCR binding domains belonging to DC8 and CIDRα1.7 belonging to group A were abundant [160]. The same group also showed using protein microarray that seroreactivity did not differ between children with cerebral or uncomplicated malaria. The antibody reactivity was found to be quite broad for all the PfEMP1 antigens tested [351].

For all three aims, dissecting the role of the different IgG subclasses involved in antibody immunity to different PfEMP1 types, identifying functional roles for the antibodies and determining the levels or titres of the measured antibodies required for conferring protection is required. Future studies, correlating the total IgG levels against the different PfEMP1 types/domains (as measured in the present thesis) with levels of functional antibodies against these PfEMP1 domains, such as inhibiting the adhesion of PfEMP1 and receptor or opsonisation could provide important insights on antibody immunity. It is

also important to confirm if the antibodies against certain PfEMP1 types (for example EPCR binders in the present study) showing potential to protect from severe malaria, can indeed lead to protection. Future longitudinal studies following malaria infected children after they have recovered from the current infection could help us answer this question.

Investigating the importance of different PfEMP1 domains in populations from Asia-pacific and Africa and narrowing down of PfEMP1 domains as immune targets for severe malaria are important, but studies have so far been impeded by the antigenic variability of PfEMP1 and small numbers of children with individual severe malaria syndromes. Although the current study found antibody responses against PfEMP1 to be non-syndrome specific in the PNG children, the study lacked the power to fully address this question. The PfEMP1 types causing severe malaria may or may not be similar to the PfEMP1 types causing the individual severe syndromes, and each of these syndromes could have a distinct molecular target of immunity [150]. Future studies examining antibody levels in a larger cohort of children with specific severe syndromes, against the PfEMP1 types identified to be associated with severe malaria in the current study, are required. These are important questions to be addressed for the development of a 'severe malaria' vaccine.

Recap of future directions

The present study has led to a better understanding of antibody responses to PfEMP1 based on disease severity. It has also helped narrow down and identify novel PfEMP1 domains as targets of immunity. This study has opened several areas of antibody immunity to PfEMP1 that can be explored further. The immediate next steps for this thesis include analysing the data from aim 1 and aim 2 together, since EPCR-binding PfEMP1 domains or types were identified as important immune targets for severe malaria in both aims. Since the same study population was used in both aims, relating the CIDR antibody profile to the opsonic antibody profile in matched individuals is required. This will help determine if the antibodies acquired against the recombinant EPCR-binding CIDR domains are reflected in differences in opsonising (functional) antibodies against parasite IE expressing the entire PfEMP1 molecule which in turn could lead to protection from severe malaria.

Validation of the recombinant proteins expressed in aim 3 by checking for their binding phenotype or comparing the cloned sequence to known databases using BLAST are required. Comparing the antibody responses against recombinant PfEMP1 domains (generated in aim 3) between Papuan adults and Malawian children and identifying the PfEMP1 domains most associated with differences in the antibody levels by age,

exposure and geography in severe malaria are also required. These types of analyses would be done using machine learning mathematical models as used in aim 3.

Having identified the specific PfEMP1 domains and domain cassettes most associated with differences in antibody responses by disease severity, the next steps would include determining the specific IgG subclasses involved in this immunity. Determining levels of functional antibodies such as opsonising antibodies (as in aim 2) against the identified PfEMP1 domains using parasite IE lines expressing the entire PfEMP1 molecule having conformations similar to the native protein and carrying the domain of interest is also required.

7.3 Conclusions

In light of recent findings linking EPCR-binding PfEMP1 to severe malaria and the importance of EPCR in cytoprotective, anticoagulative and anti-inflammatory immune functions (reviewed in [162]), antibody immunity studies to EPCR-binding PfEMP1 types or domains, such as in our study are important. Altogether, the results demonstrate an important role for antibodies against EPCR-binding PfEMP1 types or domains in PNG: antibody immunity developing after a severe infection in older children and opsonising antibodies being higher in children with uncomplicated malaria compared to severe malaria, with potential to protect from severe malaria and individual severe malaria syndromes. Thus, parasites expressing EPCR-binding CIDRα1 domains may be responsible for the pathogenesis of severe malaria in PNG. A weak evidence was also found for opsonising antibodies against ICAM-1-binding PfEMP1 (which also binds CD36) to be associated with uncomplicated malaria. Antibodies against the EPCR-binding domain could also protect from severe infections in Papuan adults. However, together with results from the Malawian study, other PfEMP1 domains present along with CIDRα1 domains (which were not all assessed in the current study) could be contributing to antibody immunity to severe malaria. Papuan adults were protected from severe infections due to a ‘total’ antibody response against a wide range of PfEMP1 domains whose transcripts were upregulated in severe malaria. In young Malawian children with cerebral malaria, the antibody responses against the PfEMP1 domains seem to be markers of exposure to antigens causing malaria rather than markers of immunity. Several novel PfEMP1 domains were identified as targets of antibody immunity to severe malaria such as DBLζ4 and CIDRα2.4.

The overall conclusion for the study is that antibody immunity to multiple PfEMP1 domains on the IE (possibly along with other VSAs- which were not tested in the present

study) may be required for protection from severe malaria and the current study has helped identify some of these PfEMP1 domains.

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9 Appendices

9.1 Appendix I: Oligonucleotide/Primer sequences used for cloning PfEMP1 domains of interest

Domain	Domain renamed	Target/transcript[a]	Primer name	Primer sequence (5'-3')
1264_X0.65	CIDRα2.4.s.1	SFM-8_S8_L001_Contig3152_frame_2	JR-SM-1-FP	TGCTCGAGCTGTCCGTGGTGTGCT
			JR-SM-1-RP	GTCCCGGGTTAGCAGTTTTGTTCTGGATTATT
1239_X0.65	CIDRβ1.s.1	SFM-3_S12_L001_Contig1763_frame_2	JR-SM-2-FP	AACTCGAGTTCTCAATTTAAAGTAAATTGTAATGG
			JR-SM-2-RP	TGCCCGGGGTTATACGTCACTATGTTGGGC
226_X0.6	DBLβ12.s.1	SFC13_S23_L002_Contig1795_frame_-3	JR-SM-3-FP	ACCTCGAGAACCCATGTATAAAACGCACT
			JR-SM-3-RP	ATCCCGGGCCTAACAACCACACGCATCTTC
96_X0.5	DBLβ3.s.1	SFC13_S23_L002_Contig1100_frm1_orf6	JR-SM-4-FP	TCCTCGAGAAATCCGTGTGTTCGTAAAGAC
			JR-SM-4-RP	TTCCCGGGTTTAACAATTACACGCATCTTCATA
1268_X0.6	DBLβ3.s.2	SFC-023_S32_L002_2405_soapGraphK61_frame_2	JR-SM-5-FP	TTCTCGAGAGGATGGGAAAATGGGAGA
			JR-SM-5-RP	AACCCGGGTCAGATATAATCATCCAATGGTGT
104_X0.75	DBLδ1.s.1	SFU-3_S21_L002_Contig2033_frame_-3	JR-SM-6-FP	AACTCGAGATGTGAAATAGTAAAAGAACTATTT
			JR-SM-6-RP	AACCCGGGATCAAGGTTTACAATTTGTTGC
177_X0.5	DBLδ1.s.2	SFM-3_S12_L001_Contig1674_frame_1	JR-SM-7-FP	GTCTCGAGCTGTCAAATAGTAAAAACACTATTT
			JR-SM-7-RP	TTCCCGGGTTTAACATGGATCACAAAGATT
262_X0.5	DBLδ1.s.3	SFM-8_S8_L001_Contig3600_frame_2	JR-SM-8-FP	ACCTCGAGTGGTGTTACAACCACTGGC
			JR-SM-8-RP	AACCCGGGGTTATGGAGCACAATATTTTGC
76_X0.85	DBLδ1.s.4	SFC19_S24_L002_Contig1569_frame_-3	JR-SM-9-FP	AACTCGAGTTGTGACATAGTTGACGAGCTA
			JR-SM-9-RP	TACCCGGGCTCAACATGGACCACAATATTTTTT
670_X0.6	DBLε2.s.1	SFC15-	JR-SM-10-FP	GACTCGAGTTCGGATAAATTGTGTAATGATAAG

		CM_S29_L002_Contig1050_frm1_orf4	JR-SM-10-RP	ATCCCGGGATCAACACGTACAGAACTTTATGAT
780_X0.6	DBLε3.s.1	SXC2_S4_L001_Contig610_frm-3_orf4	SM11.1F	CACTCGAGGTATGATAAAGGTAATGATTATATTTGT
			SM11.1R	ATCCCGGGTTCAACATTCGCATTTATTTTTGAG
1081_X0.97	DBLε9.s.1	SFM-3_S12_L001_Contig669_frame_-1	JR-SM-12-FP	ACCTCGAGTCCATTAGATAAATGTCCTTTTAGA
			JR-SM-12-RP	TTCCCGGGTTCAGCAATCACATTTTTCTTTAAG
1082_X0.9	DBLεpam5.s.1	SFM-6_S3_L001_Contig662_frame_3	JR-SM-13-FP	AACTCGAGTCCGTTAGATAGATGTTTTGAT
			JR-SM-13-RP	ATCCCGGGATCAACACTGACATTTAGAACA
348_X0.5	DBLγ3.s.1	SFM-7_S13_L001_Contig1472_frame_-1	JR-SM-14-FP	ACAAGGATCCCGTGCAAAATAGTGGATGGAATA
			JR-SM-14-RP	AAGCGGCCGCTCACTCACACTTGTCTTCAAA
371_X0.65	DBLζ4.s.1	SFU-3_S21_L002_Contig1467_frame_1	JR-SM-15-FP	CCCTCGAGTTGTGTGGAGAAAACGGCA
			JR-SM-15-RP	GCCCCGGGCTAGCATTTACATTTATCTTTATACTC
350_X0.65	DBLα0.13.ns.1	IFM23_S4_Contig695_frame_-2	JR-UM-01-FP	ATCTCGAGATGCGAACTTGTAAAACAATAT
			JR-UM-01-RP	TCCCCGGGATCAACATGCTTTGCAATATTT
123_X0.5	DBLδ1.ns.2	IFM047_S10_Contig953_frame_3	JR-UM-02-FP	AACTCGAGATGTGAAATAGTAAAAGACCTATTT
			JR-UM-02-RP	TTCCCGGGTTCAACATGGTTTACAATTTGT
479_X0.5	CIDRα2.5.ns.1	IFM23_S4_Contig695_frame_-2	JR-UM-03-FP	TGCTCGAGATGTCCGTGGTGCGGA
			JR-UM-03-RP	ACCCCGGGCTCATTTGTGTGTTTTTAGGCAGTT
345_X0.5	DBLγ13.ns.1	IFM054_S12_Contig1100_frame_-2	JR-UM-04-FP	AACTCGAGGTGCGAAATAGTGGAAGAGGTT
			JR-UM-04-RP	TGCCCGGGGTCAAGTACACTTATTTTCGTATCC
523_X0.7	DBLα0.11.ns.1	IFM27_S9_Contig420_frm1_orf3	JR-UM-06-FP	TGCTCGAGTGATTATACTACACGTTTTGATGCT
			JR-UM-06-RP	TGCCCGGGATCAACATGTATCGCAATATTCCTT
341_X0.5	DBLγ9.ns.1	IFM12_S16_Contig851_frame_-3	JR-UM-08-FP	ACCTCGAGGTGCAAAATGGTGCAGAAATTA
			JR-UM-08-RP	ATCCCGGGCTCAATTACACTTCCCTTTGAC
551_X0.5	CIDRβ1.ns.1	SFU2_S5_L001_Contig1892_frame_1	JR-UM-09-FP	GACTCGAGTTCTGAATTTAAAATAAATTGTAATAAT
			JR-UM-09-RP	TTCCCGGGGTCATTGGGTTTGGGTTTC

83_X0.5	DBLδ1.ns.6	IFD6_S1_Contig745_frm1_orf15	JR-UM-14-FP	GTCTCGAGTTGCGGCATAGTAAACACA
			JR-UM-14-RP	TTCCCGGGCTCAACATGTACCACAATTTTC
310_X0.5	DBLα0.3.ns.1	SFU2_S5_L001_Contig1725_frame_-2	JR-UM-18-FP	GACTCGAGATGCGCCCTTAATTATACATATGAT
			JR-UM-18-RP	TCCCCGGGGTCAACATGGTTTACAATATTGCGA
60_X0.6	DBLδ1.ns.3	SFM-10_S1_L001_Contig2583_frame_-2	JR-UM-19-FP	GTCTCGAGATGTGATATAGTAAACACACTATTT
			JR-UM-19-RP	TTCCCGGGCTCAACATGGATCACAATTTTT
486_X0.5	CIDRα3.1.ns.1	IFM12_S16_Contig843_frm2_orf3	JR-UM-20-FP	TGCTCGAGCTGTCCTTATTGTGGAATGAAA
			JR-UM-20-RP	GCCCCGGGCCTATTTTTCTAGGCATTTGGT
447_X0.6	DBLα0.9.ns.1	IFM047_S10_Contig1232_frame_-3	JR-UM-21-FP	TCCTCGAGATGCGAACTTGATTATAATTTTCAT
			JR-UM-21-RP	ACCCCGGGGTCATGCTTGACAATATTTTGTACT
45_X0.5	DBLδ1.ns.5	IFM047_S10_Contig984_frame_-3	JR-UM-33-FP	GTCTCGAGATGTCAAATAGTAAAAACACTATTTGAA
			JR-UM-33-RP	AGCCCGGGGTCAACAATTTGTTGCAGGTCT
398_X0.97	DBLpam2.ns.1	IFM047_S10_Contig664_frm-2_orf1	JR-UM-43-FP	CACTCGAGATGTGAAAAAAATTTAGATGAAGCC
			JR-UM-43-RP	AGCCCGGGTTCAACACTCACAAACATATTTATATTC
1297_X0.65	CIDRα1.7.ns.1	IFM23_S4_Contig698_frm-1_orf23	JR-UM-45-FP	CACTCGAGTCCCCATTGTGGAGTCGAT
			JR-UM-45-RP	CTCCCGGGTTCAACATGCCTCGTTTGTATT
411_X0.6	DBLα0.11.ns.2	IFC66_S31_L002_5262_soapGraphK61_frame_1	JR-UM-52-FP	AACTCGAGATGCGAACTTGAATATAAATATCAT
			JR-UM-52-RP	ACCCCGGGGTCATGCTTGACAATATTTTGTATG
	DBLα1.2	SFC17_S7_L001_Contig1006_frm2_orf5	SM-16F	GGCTCGAGGTGTAAGCTTAGTCACAAATTCCAT
			SM-16R	GCCCCGGGGTCACACTTGGCAATATTCTGA
	DBLα1.5	SFM-3_S12_L001_Contig1068_frm1_orf6	SM-17F	GACTCGAGATGCGAACTTGATTTTGCTTTT
			SM-17R	ACCCCGGGGTCAACACGTTTCACAATATTC
	CIDRα1.1	SFM-8_S8_L001_Contig3547_frame_1	SM-18F	CACTCGAGTCCCGATTGTGTAGTTAAATGC
			SM-18R	GCCCCGGGCCTATGCTAATGAATTATTATTTATGCA

	CIDRα1.6	SFM-10_S1_L001_Contig1304_frm-3_orf10	SM-19F	GACTCGAGTCCATTTTGTGGAGTTGAATGT
			SM-19R	CTCCCGGGCCTAACATGATTCGTTTGAATTATT
	DBLγ11	SFM-10_S1_L001_Contig1304_frm-3_orf10	SM-20F	GACTCGAGGTGCGAAATAGTGGATGGA
			SM-20R	TGCCCGGGGTCAACAATTACATTTACCTTTGAC
	DBLα2	SFM-8_S8_L001_Contig3547_frame_1	SM-21F	TTCTCGAGGTGTGACATTAATGATAAATTTGAA
			SM-21R	ACCCCGGGGTCAGGGTTGGCAATAATC
	DBLε5	SFC-023_S32_L002_Contig803_frame_2	SM-22F	ACCTCGAGTCCATTGGATAAATGTCCTTTCAAT
			SM-22R	TTCCCGGGATCATGAACCAGGATATAATTTTTTTAG
	CIDRγ9	SFC19_S24_L002_Contig1562_frame_-1	SM-23F	AACTCGAGTCCTATATATGGAGTTAAGTGTAAT
			SM-23R	TTCCCGGGACTAGGTACCATTATGTTTCGA
	DBLζ3	SFC14_S17_L001_Contig2402_frm-3_orf12	SM-24F	CCCTCGAGTTGTATAGACAAAGCTGCATATGAA
			SM-24R	AGCCCGGGTTCAAACACAATGACTTTTATACAT
	DBLβ13	SFU-3_S21_L002_Contig2030_frame_-3	SM-25F	AACTCGAGAAATCCATGTAGTGGCGAC
			SM-25R	CTCCCGGGCCTAACACTTACACGCACC
	CIDRγ12	SFC14_S17_L001_Contig441_frm2_orf2	SM-26F	GACTCGAGTCCTATATATGGAGTTAAGTGTAAT
			SM-26R	TTCCCGGGCCTACTGTGTGTTTTTGCATTC
	DBLδ7	SFC19_S24_L002_Contig1853_frame_2	SM-27F	CGAAGGATCCTGTGCACAACAGTAGAACAACTT
			SM-27R	AACGCGGCCGCGTCAACATGGTTTACAATTATCTG C
	CIDR2.6-DBLβ5	SFC13_S23_L002_Contig2410_frame_1	SM-28F	GACTCGAGTCCGTGGTGTGGAGCC
			SM-28R	TTCCCGGGACTATTCTATTATCTTTTTTGCTTCGTG
	Ama-1	Plasmodb_Predicted RNA/mRNA Sequence (Introns spliced out)	AMA1-F	ATCTCGAGATTATACTGCGTATTATTATTGAGC
			AMA1-R	ATCCCGGGGTTATTCCATCAGAACTGGTGT
	CSP-1	Plasmodb_Predicted RNA/mRNA Sequence (Introns spliced out)	CSP1-F	ATCTCGAGAAAATTAGCTATTTTATCTGTTTCTTCC
			CSP1-R	ATCCCGGGACTAGAAGGATAATACCATTATTAATC

C

CTCGAG, XhoI restriction binding site incorporated in the forward primer

CCCGGG, XmaI restriction binding site incorporated in the reverse primer

GGATCC, BamHI restriction binding site incorporated in the forward primer

GCGGCCGC, NotI restriction binding site incorporated in the reverse primer

[a] Patient id/ sample used as template for amplification.

9.2 Appendix II: Oligonucleotide/Primer sequences used for sequencing the successfully cloned domains of interest

Domain	Domain renamed	Target/transcript	Primer name	Primer sequence (5'-3')
		pEU-E01-His-TEV-MCS-N1	JR-pEU 2-FP	TTAGGTGACACTATAGAACTCACC
			JR-pEU 2-RP	TCTTAAACACAGCCTGATATAGGA
1264_X0.65	CIDRα2.4.s.1	SFM-8_S8_L001_Contig3152_frame_2	SM01INT-F1	TGGAGTAAAGAACTTGATAGGTGT
1239_X0.65	CIDRβ1.s.1	SFM-3_S12_L001_Contig1763_frame_2	SM02INT-F1	GATGGTACATACATTATACAAATTAGAGCA
226_X0.6	DBLβ12.s.1	SFC13_S23_L002_Contig1795_frame_-3	SM03INT-F1	GGAGATTTCATAGTAGAAAAACTTAAA
			SM03INT-R1	CTTACAATACCATTCTGCCCATTC
96_X0.5	DBLβ3.s.1	SFC13_S23_L002_Contig1100_frm1_orf6	SM04INT-F1	ATCAGGTTCAATATAGGAGAAAATTGG
			SM04INT-F2	TGGGATCAAAATATTGGAGAACAA
			SM04INT-R1	AATTGGCATTTCCTGATGTATGTA
			SM04INT-R2	TGCTCTTATTTTATCCCATTGAGG
1268_X0.6	DBLβ3.s.2	SFC-023_S32_L002_2405_soapGraphK61_frame_2	SM05INT-F1	ATTTTTAGTAAAATTAAAGAGAAAGTTCCT
104_X0.75	DBLδ1.s.1	SFU-3_S21_L002_Contig2033_frame_-3	SM06INT-F1	AGACGACGATTATACGTAACTCCA
			SM06INT-F2	TTACAAAGTGGTACCATACCCACC
			SM06INT-R1	GTTACCATTATTACGTGTAGCATC
			SM06INT-R2	GGGGCGTACCACAAAATTTTT
262_X0.5	DBLδ1.s.3	SFM-8_S8_L001_Contig3600_frame_2	SM08INT-F1	TCACCAGCAGCTGGAATACAA
			SM08INT-F2	GACGAAAAACGTAAAAAATGGTGG
			SM08INT-R1	TTCATTACCATTATTATTACTTGTAGCATC
76_X0.85	DBLδ1.s.4	SFC19_S24_L002_Contig1569_frame_-3	SM09INT-F1	CCCAGGAGGAGACGATTATAT
			SM09INT-F2	GGTTCAGGCAGTGATGATAGT
			SM09INT-R1	TCCTAATGTTTTAGAAAATTCTTTGTAATC

			SM09INT-R2	CCACTCATGTAACCAACGAAA
780_X0.6	DBLε3.s.1	SXC2_S4_L001_Contig610_frm-3_orf4	SM11.1INT-F1	GAAAAACGTAAAAAATGGTGGGAC
1081_X0.97	DBLε9.s.1	SFM-3_S12_L001_Contig669_frame_-1	SM12INT-F1	AAAGATCAACCTACAAAAGCATTA
			SM12INT-R1	AATAAAATCTGGCTTTTTCTTACTATT
1082_X0.9	DBLεpam5.s.1	SFM-6_S3_L001_Contig662_frame_3	SM13INT-F1	ATTGGAGATGCTATAGGATGTAAGGAT
			SM13INT-R1	TGATTTAACGTATTCTTTATGCTCTTG
348_X0.5	DBLγ3.s.1	SFM-7_S13_L001_Contig1472_frame_-1	SM14INT-F1	TGTTTGGATAAAAATATAGGTAATGATGTG
			SM14INT-R1	ACTGTATGCATCTTCTACATCTTC
371_X0.65	DBLζ4.s.1	SFU-3_S21_L002_Contig1467_frame_1	SM15INT-F1	TATTGCAATAATGTCACTAAAGAAACA
			SM15INT-F2	GCCAAAAATGAAGGCGATGATATAATA
			SM15INT-R1	TTCACATTTTGTTTTGCATTTTTCACA
350_X0.65	DBLα0.13.ns.1	IFM23_S4_Contig695_frame_-2	UM01INT-F1	ACGTCGACGAAGAATGATTTG
			UM01INT-F2	CAAAAACGCTACGAAGGTGATGAT
			UM01INT-R1	ATTTTTTTCAAGTTTGTCATAAAATTTTTT
123_X0.5	DBLδ1.ns.2	IFM047_S10_Contig953_frame_3	UM02INT-F1	GCTGCTGTGGAAACTTTTTTCTTG
			UM02INT-F2	AAAAAACCTGGTGGTGACAAAAAT
			UM02INT-R1	GTTTTTTTGTCCAGTATATGCCTC
479_X0.5	CIDRα2.5.ns.1	IFM23_S4_Contig695_frame_-2	UM03INT-F1	GGTGGTGCTGCAAATAGAACT
			UM03INT-R1	CTCAATAATTTCCAAAAGTTCTTCTTT
523_X0.7	DBLα0.11.ns.1	IFM27_S9_Contig420_frm1_orf3	UM06INT-F1	GAAACCGAAAGAGAAAAATTAGAAAATAAG
			UM06INT-R1	TTGTAGTTGTTGATAAAATTCTTTTACATA
341_X0.5	DBLγ9.ns.1	IFM12_S16_Contig851_frame_-3	UM08INT-F1	GAAAACTGGTGGAAAGAAAATGGT
			UM08INT-R1	TTTATCTAAATAATCGCGAGCATC
83_X0.5	DBLδ1.ns.6	IFD6_S1_Contig745_frm1_orf15	UM14INT-F1	CAGTCACCAAATGGTGACTTG
			UM14INT-F2	GTGAGTGCTAGTGGTAACACAAAT

			UM14INT-R1	GAGACTTTTTAGTGTTGCACAAAATTC
			UM14INT-R2	CCATTCTTCAAGGTATCGGAAATA
310_X0.5	DBLα0.3.ns.1	SFU2_S5_L001_Contig1725_frame_-2	UM18INT-F1	CAAAAAAAAAATGAATTAGAAGCAAGTTTC
			UM18INT-R1	TTCTGCATTTAACAAATTTATAAATTCATG
60_X0.6	DBLδ1.ns.3	SFM-10_S1_L001_Contig2583_frame_-2	UM19INT-F1	GAAAAAGCACGACAAAATGGATTG
			UM19INT-F2	AATGATGAGAAAAAAGCCGCTAGT
			UM19INT-R1	ACCACCCTTATTATTTCCATTTAC
			UM19INT-R2	TTCTTCAAGGTATCGGAAGTAGGG
486_X0.5	CIDRα3.1.ns.1	IFM12_S16_Contig843_frm2_orf3	UM20INT-F1	TGCAAAACAGAAGATGATGAATCTTTA
			UM20INT-R1	TTCCTTATCTTCTTCAGTATTTTCTTTAGA
447_X0.6	DBLα0.9.ns.1	IFM047_S10_Contig1232_frame_-3	UM21INT-F1	GGTGAATGTGCTGATAGTAAAATAAAA
			UM21INT-F2	GGAAATAAAAAAAAAAATCAAGCAAGA
			UM21INT-R1	TGGATAATATACATGCAGTATTTTATAAAA
			UM21INT-R2	ACGACATTTTTCTATAGCATTTTCTAATTT
45_X0.5	DBLδ1.ns.5	IFM047_S10_Contig984_frame_-3	UM33INT-F1	GAGACACAAACAAGTGGTAGT
			UM33INT-F2	TTCTTGCGTCAAATGTTTTATACTTTA
			UM33INT-R1	GTTATTACGTTCAGCAGCATTACA
			UM33INT-R2	TTCTTCAAGGTATCGGAAGTAGGT
398_X0.97	DBLpam2.ns.1	IFM047_S10_Contig664_frm-2_orf1	UM43INT-F1	AAATGGATATGGAAAAAATACTCTGGT
			UM43INT-R1	CTTTACATCTTCGCACACATTTTC
			UM43INT-R2	ACTACTTGGGCCACAATTTTTTGT
			UM43INT-R3	TGCTTGACGTTGTTTACAAAAATGTTC
1297_X0.65	CIDRα1.7.ns.1	IFM23_S4_Contig698_frm-1_orf23	UM45INT-F1	TCTAACAAATTAAGTGAATTTTGTAATGAG
			UM45INT-R1	TTCGTTAAGCTCATACATAACTTG
411_X0.6	DBLα0.11.ns.2	IFC66_S31_L002_5262_soapGraphK61_frame_	UM52INT-F1	GTGTGTCTTGCAGCAAAATTTGAA

		1	UM52INT-F2	TATCAATTACGTGAAGATTGGTGGAAC
			UM52INT-R1	TTGTTCATAAAATTCTTTTACATACAAGTT
	DBLα1.5	SFM-3_S12_L001_Contig1068_frm1_orf6	SM17INT-F1	GGGAATGAAAAAGATCAATTAGAAAATAAC
			SM17INT-R1	ATAGAATTCTTGAACATATTTATTTTCGTC
	DBLγ11	SFM-10_S1_L001_Contig1304_frm-3_orf10	SM20INT-F1	TATAGGGATTTATGTTTGGATAAAAATATA
			SM20INT-R1	TAACTCTTTTGCGTCGGAATC
	DBLε5	SFC-023_S32_L002_Contig803_frame_2	SM22INT-F1	ATGTTATTGGGGTATCAAAAAGGTAAA
	DBLζ3	SFC14_S17_L001_Contig2402_frm-3_orf12	SM24INT-F1	AAATTAGAAACGATTTTCAACAAAATA
			SM24INT-R1	AAATTCAATAACATGTTTATCGCTATC
	DBLβ13	SFU-3_S21_L002_Contig2030_frame_-3	SM25INT-F1	CTAAATAATGAGAAAACTGTATGTCGT
			SM25INT-R1	GGCAACACTTGATGTGTGTAATTT
	CIDRγ12	SFC14_S17_L001_Contig441_frm2_orf2	SM26INT-F1	ACTACATTTAAAGTATTGCTAATATATTGG
	DBLδ7	SFC19_S24_L002_Contig1853_frame_2	SM27INT-F1	AGAGGAAAAATACCTGAAGAGTTTAAG
			SM27INT-R1	ACCACCCTTACTTCCCTTTTC
	CIDRα2.6-DBLβ5	SFC13_S23_L002_Contig2410_frame_1	SM28INT-F1	TTGTGTGAAAAATGGATATGTTATTAT
			SM28INT-F2	ATAAAAAAACATTTTGACACGCAAGTT
			SM28INT-R1	TTTCCATTCCGTTCCTATTTCAAA
	Ama-1	plasmodb_Predicted RNA/mRNA Sequence (Introns spliced out)	AMA1INT-F1	GATGGAGGTTTTGCTTTTCCT
			AMA1INT-F2	TTTTGTTTTAGACCAGCAAAAGAT
			AMA1INT-R1	TAAAATAGTTGCTAATACAGCGAC
			AMA1INT-R2	AATTCGTTTTGATTCTCTTTCGAT
	CSP-1	plasmodb_Predicted RNA/mRNA Sequence (Introns spliced out)	CSP1INT-F1	AAATTAAAGCAACCAGCGGAT
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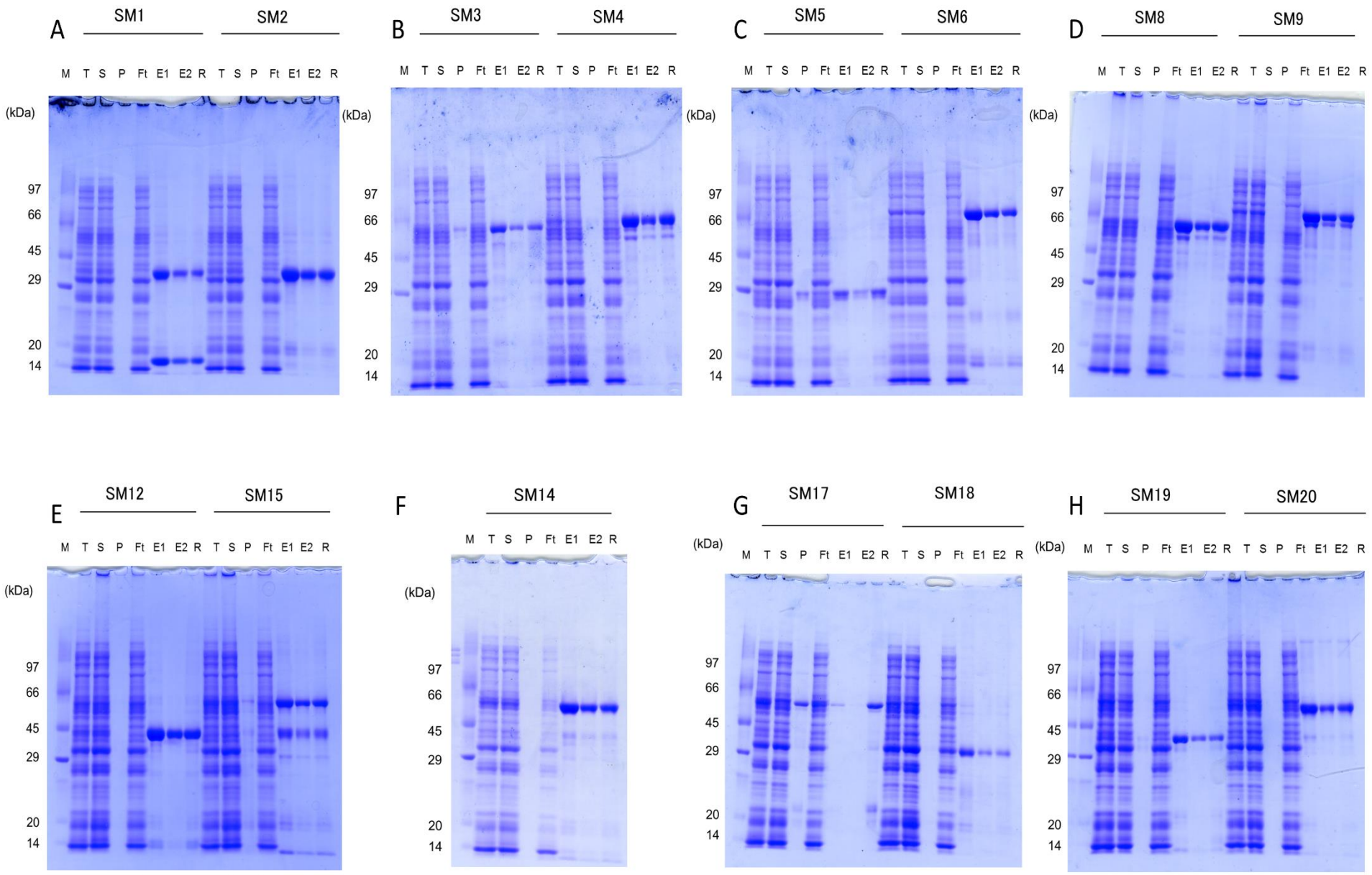
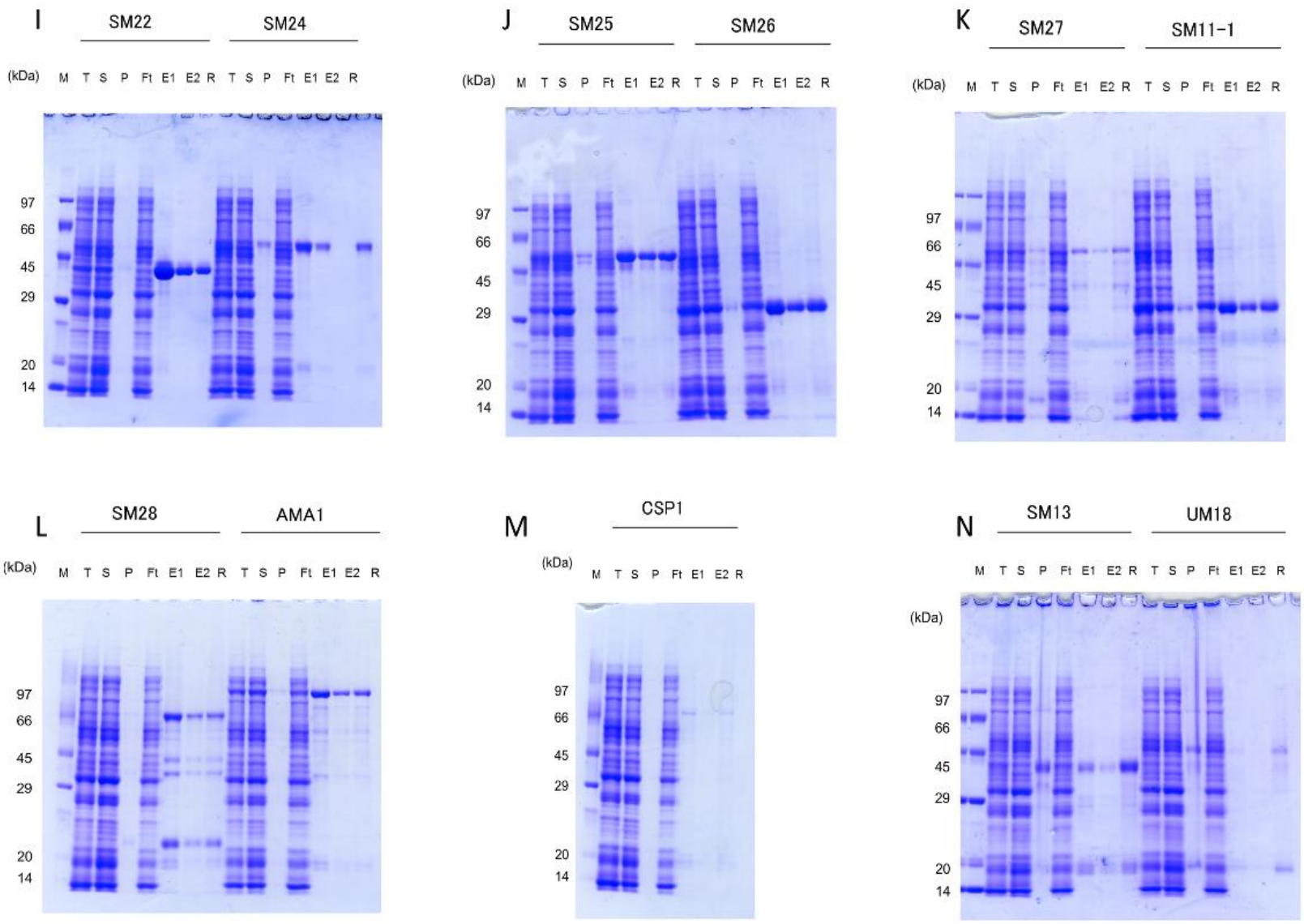
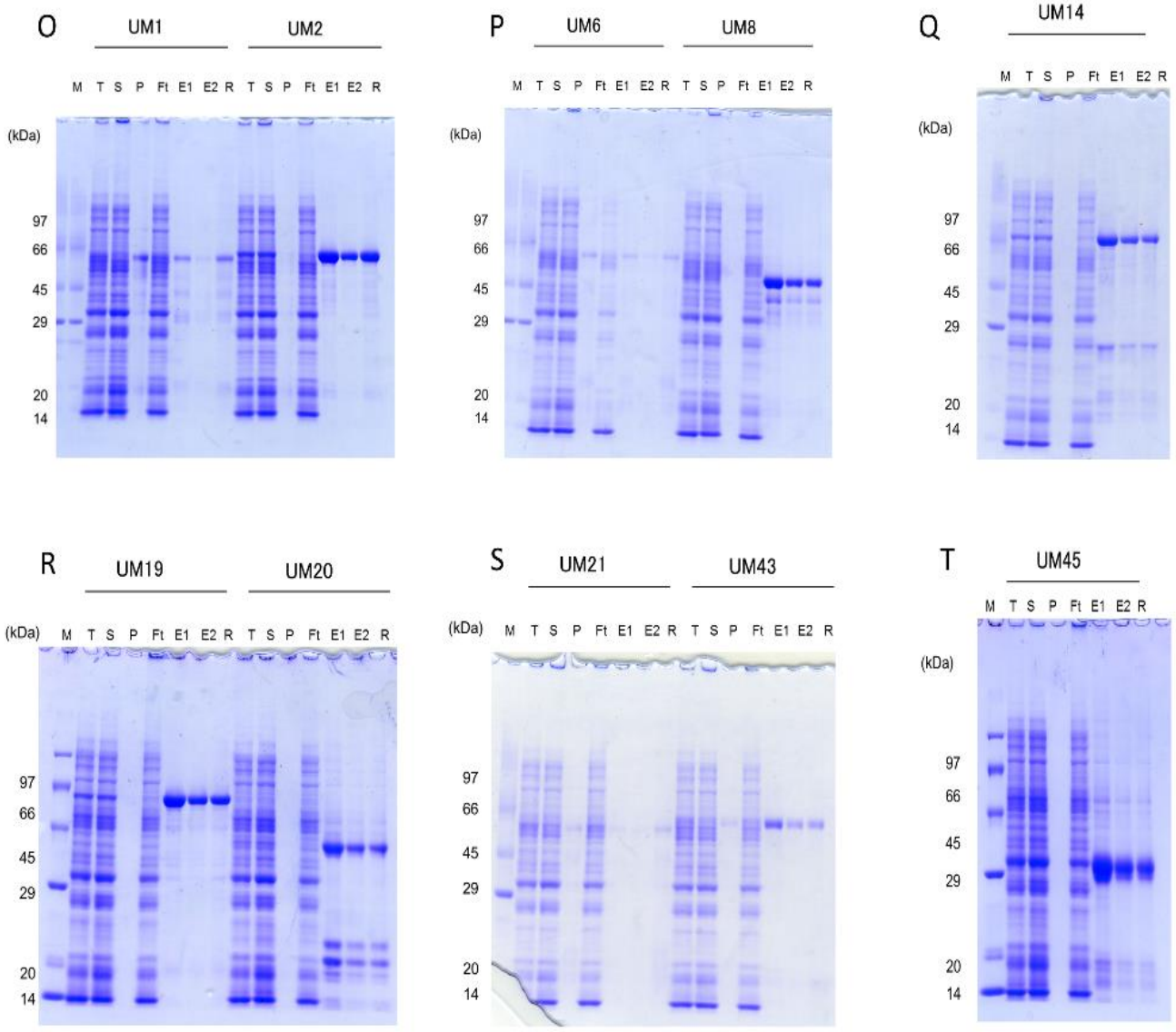
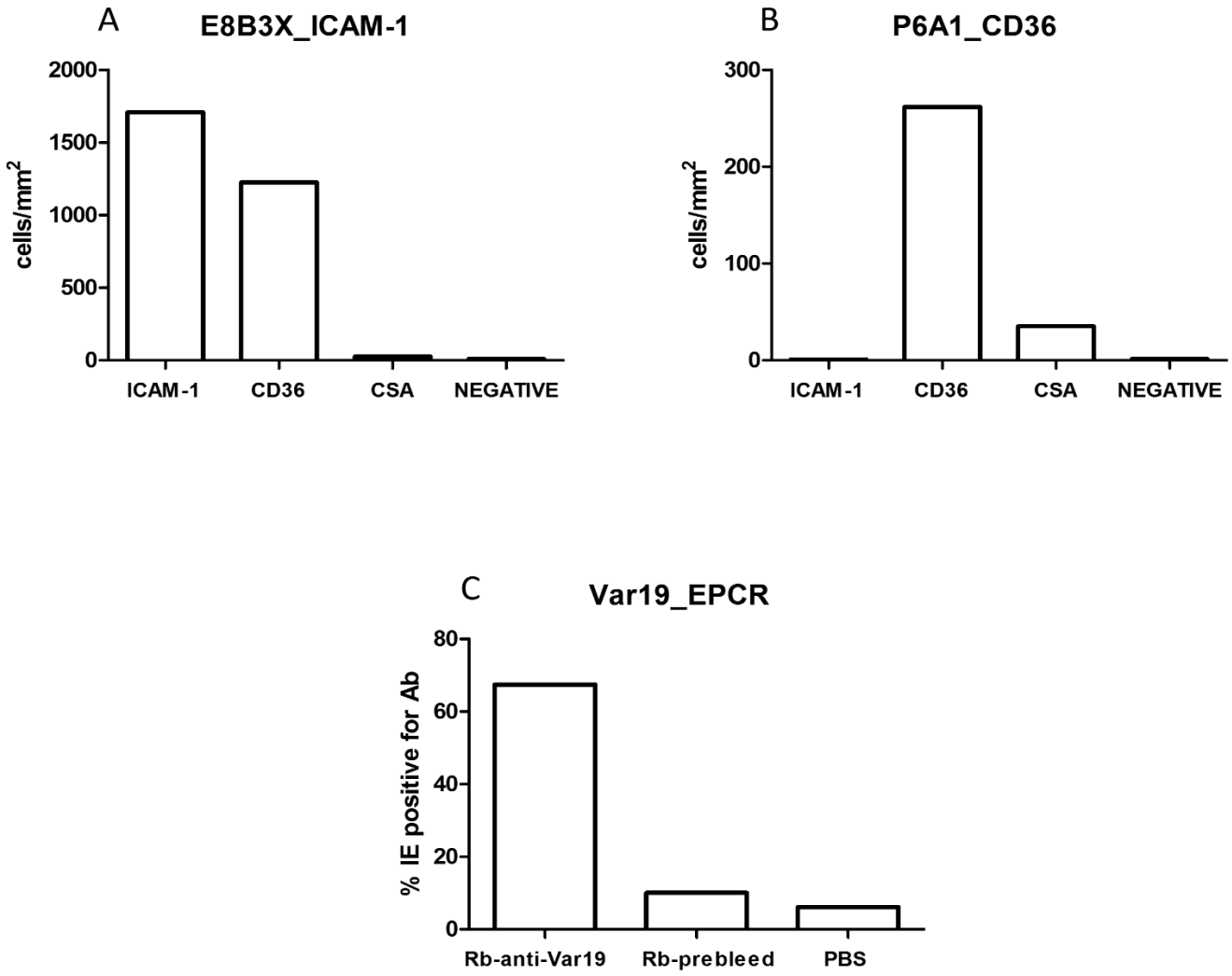

A
SM1
SM2
B
SM3
SM4
C
SM5
SM6
D
SM8
SM9
E
SM12
SM15
F
SM14
G
SM17
SM18
H
SM19
SM20
M T S P Ft E1 E2 R T S P Ft E1 E2 R
(kDa)
97
66
45
29
20
14


I
SM22
SM24
(kDa)
M T S P Ft E1 E2 R T S P Ft E1 E2 R
97
66
45
29
20
14
J
SM25
SM26
(kDa)
M T S P Ft E1 E2 R T S P Ft E1 E2 R
97
66
45
29
20
14
K
SM27
SM11-1
(kDa)
M T S P Ft E1 E2 R T S P Ft E1 E2 R
97
66
45
29
20
14
L
SM28
AMA1
(kDa)
M T S P Ft E1 E2 R T S P Ft E1 E2 R
97
66
45
29
20
14
M
CSP1
(kDa)
M T S P Ft E1 E2 R
97
66
45
29
20
14
N
SM13
UM18
M T S P Ft E1 E2 R T S P Ft E1 E2 R
(kDa)
97
66
45
29
20
14


O
UM1
UM2
M T S P Ft E1 E2 R T S P Ft E1 E2 R
(kDa)
97
66
45
29
20
14
P
UM6
UM8
M T S P Ft E1 E2 R T S P Ft E1 E2 R
(kDa)
97
66
45
29
20
14
Q
UM14
M T S P Ft E1 E2 R
(kDa)
97
66
45
29
20
14
R
UM19
UM20
(kDa) M T S P Ft E1 E2 R T S P Ft E1 E2 R
97
66
45
29
20
14
S
UM21
UM43
(kDa) M T S P Ft E1 E2 R T S P Ft E1 E2 R
97
66
45
29
20
14
T
UM45
M T S P Ft E1 E2 R
(kDa)
97
66
45
29
20
14

9.3 Appendix III: SDS gel images for the proteins expressed using WGCFS.

A-T**:** The images are for the 23 PfEMP1 domains upregulated in severe malaria and 11 domains upregulated in uncomplicated malaria. SM20 (panel H) and UM18 (panel N) were not expressed successfully. The protein expression was done by Dr. Eizo Takashima and Professor Takafumi Tsoboi from Ehime University, Matsuyama, Japan. In the figure, M: Marker (in kDa), T: total translation mix, S: supernatant, P: pellet, Ft: flowthrough, E1: elution 1, E2: elution 2, R: resin. SDS, sodium dodecyl sulphate; WGCFS, wheat germ cell free synthesis systems; SM, severe malaria; UM, uncomplicated malaria.


A
E8B3X_ICAM-1
cells/mm2
2000
1500
1000
500
0
ICAM-1
CD36
CSA
NEGATIVE
B
P6A1_CD36
cells/mm2
300
200
100
0
ICAM-1
CD36
CSA
NEGATIVE
C
Var19_EPCR
% IE positive for Ab
80
60
40
20
0
Rb-anti-Var19
Rb-prebleed
PBS

9.4 Appendix IV: Binding phenotypes for E8B3X_ICAM-1, P6A1_CD36 and Var19_EPCR IE lines used for opsonic phagocytosis assay.

The relative binding of A) E8B3X_ICAM-1 and B) P6A1_CD36 to the receptors: ICAM-1, CD36, CSA along with the negative (no receptor) control was measured using a receptor binding assay. The binding is represented as cells/mm^2 on the Y-axes while the X-axes represent the receptors. See section 3.2.9 for details of the binding assay. C) For Var19_EPCR, the PfEMP1 phenotype (using a variant surface antigen assay) after sorting with rabbit anti-Var19 antibody is presented. Rabbit-preebleed (non-immune serum) and PBS were the controls. Y-axis represents the percentage of infected erythrocytes positive for antibody. X-axis represents the different antibodies used in the PfEMP1 phenotype assessment assay. Var19 phenotype assessment was done by Ms. Wina Hasang. Each antibody was tested in duplicates and an average of the duplicates is presented here. IE, infected erythrocytes; Ab, antibody; PBS, phosphate buffer saline.