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Major article

Running head: Antibodies to severe malaria in PNG

Title

Acquisition of antibodies against Endothelial Protein C Receptor-binding domains of *P. falciparum* erythrocyte membrane protein 1 in children with severe malaria

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Abstract

Background

Plasmodium falciparum erythrocyte membrane protein-1 (PfEMP1) mediates parasite sequestration in postcapillary venules in *P. falciparum* malaria. PfEMP1s can be classified based on their cysteine-rich inter-domain region (CIDR) domains. Antibodies to different PfEMP1 types develop gradually following repeated infections as children age, and antibodies to specific CIDR types may confer protection.

Methods

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

Results

At baseline, antibody levels were similar across the three groups. Following 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, but not 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 PNG.

Key words

PfEMP1, *Plasmodium falciparum,* severe malaria, antibodies, Papua New Guinea, luminex, CIDR, EPCR

40-word summary

Papua New Guinean children with severe malaria acquired antibodies against EPCR-binding CIDRα1 domains of *Plasmodium falciparum* erythrocyte membrane protein 1 during convalescence. Antibody levels increased from clinical presentation to convalescence in older children with severe, but not uncomplicated, malaria.

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Introduction

Malaria caused 429,000 deaths in 2015, mainly due 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, multi-domain adhesins, which are exported to the IE surface and exposed to host antibodies [6]. Encoded by a repertoire of around 60 *var* genes per parasite genome, PfEMP1s mediate adhesion to host receptors, including intercellular adhesion molecule-1 (ICAM-1) [7], CD36 [8], complement-receptor 1 (CR1) [9], chondroitin sulphate A (CSA) [10] or 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 (DBL) domains, and cysteine-rich inter-domain 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, whilst 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 promotor sequences (UPSA-C), transcriptional direction and chromosomal location [21]. Domain cassettes (DC) 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 one or two 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 high malaria transmission areas [29].

Var gene expression studies 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 *P. 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 PfEMP1s [35]. Group B *var* transcription was higher than Groups A or C *var* transcripts in children with severe or uncomplicated malaria while Group C transcription was elevated in asymptomatic children [35]. Recently, RNA-seq 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], while antibodies from individuals with uncomplicated malaria recognised 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. Further, antibodies to PfEMP1 expressed on IE (DC8 and DC13 variants) were higher in uncomplicated malaria than severe malaria, suggesting these antibodies could protect from severe malaria (Chan JA et al., submitted to Journal of Infectious Diseases).

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 [38]. Severe malaria was defined based on World Health Organization (WHO) criteria [39], including a BCS (Blantyre Coma Score) <5 or impaired consciousness; severe anaemia (haemoglobin <50 g/L); respiratory distress; prostration; multiple seizures; hyperlactataemia (blood lactate > 5 mmol/L); dark urine; hypoglycaemia (blood glucose ≤ 2.2 mmol/L); jaundice; abnormal bleeding; persistent vomiting; or signs of shock [38]. Key severe malaria presentations are summarised in Supplementary table 1; children with uncomplicated malaria had no criterion for severe malaria. Healthy asymptomatic children from Madang region were recruited as community controls. Controls were matched by age (±12 months), gender and ethnicity. Spleens were palpable in 13% and prevalence of *P. falciparum* was 8.2% and *P. vivax* was 14.1% by microscopy. *P. falciparum* infection was confirmed by light microscopy and speciation by nested polymerase chain reaction (PCR) [38].

At presentation, venous blood was collected and plasma was separated by centrifugation and stored at -70^{o}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* infections. Four children with uncomplicated malaria lacked complete clinical information, 19 had *P. vivax* infections, 7 did not have parasite densities recorded and 17 did not have PCR 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 Number 1103) and the Medical Research Advisory Committee of the PNG Health Department (MRAC Number 11.12). The study was conducted as per the principles of the Declaration of Helsinki [38].

Luminex assay and measurement of IgG levels

35 recombinant HIS-tagged CIDR domains (Supplementary table 2) were expressed in Drosophila Sf9 cells and purified by nickel affinity chromatography as previously described [18, 29]. Total IgG levels against these proteins were measured using a published luminex assay [40], with minor modifications. Briefly, plasma samples were diluted 1:160 in Assay Buffer E (ABE: 0.1% BSA, 0.05% Tween-20 in PBS, pH7.4). In ABE, a ten point, two-fold dilution of pooled positive plasma starting with a dilution of 1:40 was carried out. Equal

volumes of beads (initial concentration 1.25 X 10^7 microspheres/ml) coupled to each domain of interest were mixed, and then diluted 1:333 in ABE. 50 µl of beads and 50 µl of diluted plasma were added to 96-well microtiter plates (MSBVS 1210, Millipore, USA) pre-wetted with ABE. 50 µl of phycoerythrin-conjugated Goat Anti-Human IgG (Jackson ImmunoResearch Laboratories), diluted 1:3500 was added and using the BioPlex^{100} system, mean fluorescent intensities and antibody concentrations were measured. 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 1.4-1.8, N=19) and sub-divided into Group A (CIDRα1.4-1.7, N=13) and Group B/A (CIDRα1.1 and 1.8, N=6); those binding CD36 [19] (CIDRα2-6, N=12) and those binding neither EPCR nor CD36 [29] (CIDRδ, N=3; CIDRγ, N=1). Mean antibody levels to these proteins were compared among and within the different sample groups (severe acute, uncomplicated acute, severe convalescent, uncomplicated convalescent and community controls), across different age groups and against each of the CIDR domain types.

STATA13 and GraphPad PRISM5 softwares 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 analysed 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, uncomplicated malaria, and community controls (Fig 1). The exception was for antibody to CD36-binding CIDR domains, which was higher in uncomplicated malaria than community controls (p = 0.0109) (Fig. 1).

When antibody levels against individual domains were compared, children with severe malaria had higher levels of antibody than those with uncomplicated malaria against CIDRα1.5b(b). Children with uncomplicated malaria had higher antibody levels 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 (Fig. 2). In older children, antibody levels were similar at study entry in all three groups (Fig. 2). These data probably 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 following severe or uncomplicated malaria

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

Convalescent antibody levels against seven individual CIDRα1 domains and also against CIDRα2.9 were significantly higher in children with severe malaria than children with uncomplicated malaria. These variants included multiple CIDRα1.1, CIDRα1.5, 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 (Fig. 4A, 4B, 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 (Fig. 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 (Fig. 4).

Comparing the antibody levels between younger and older children following severe malaria or uncomplicated malaria separately, we found higher antibody levels to EPCR-binding CIDRs in older children than in younger children following severe malaria. Such differences in antibody levels were not observed in children convalescent from uncomplicated malaria (Supplementary fig. 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 than acute plasma. 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 non-significant) (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, where younger children showed a decrease in antibody levels, $p = 0.030$) (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 and without severe anaemia, respiratory distress, metabolic acidosis, hyperlactataemia, and 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 antibody levels 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).

In convalescent plasma, children with impaired consciousness (BCS score 0-4) had higher antibody levels against Group B/A CIDR EPCR binders than children with uncomplicated malaria (Supplementary figure 4). No differences in antibody levels were observed on comparing children with cerebral malaria (BCS score ≤ 2) to children with impaired consciousness (BCS score 3, 4) or with 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], 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 than matched children with severe or uncomplicated malaria or than older community controls against most CIDR domain types, possibly reflecting their lack of exposure to malaria [41]. The antibody reactivity detected at presentation could result from either previous or ongoing infections.

In convalescence, children with severe malaria had higher antibody levels than the community controls against EPCR-binding CIDRα1 domains, and higher than the uncomplicated malaria group for the Group B/A (DC8) CIDRα1 domain types. On stratifying by age, older children with severe malaria had higher antibody levels to EPCR-binding CIDRα1 domains belonging to Group A and Group 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 stratifying by age are in line with a recent Ugandan study demonstrating strong associations between increasing age and anti-parasite (density) and anti-disease (parasite density with fever) immunity after controlling for exposure. Maturation of the immune system could also explain these age-stratified antibody responses [42]

Altogether, these data suggest that antibodies against EPCR-binding domains develop most prominently following 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 anaemia had limited antibody repertoires against non-CD36 binding PfEMP1 at presentation, and convalescent sera gave more intense responses than acute sera to non-CD36 binding PfEMP1s and PfEMP1s containing CIDRα1 domains. In that study, sera reactivity was measured against domains of CIDRα1-containing PfEMP1s and not always the CIDRα1 domain itself [43]. Our results are consistent with a study in Papuan adults, showing that protection from severe malaria was associated with exposure to a broad range of Group A and B PfEMP1s, while people 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 pre-existing antibodies in participants [44].

In older children with uncomplicated malaria antibody levels decreased in convalescent plasma against the EPCR-binding CIDRα1 domain types. In children with uncomplicated malaria from Kilifi, antibody responses to surface antigens also declined over twelve weeks following malaria episodes [45]. 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 [45] 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 PfEMP1s 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, as a putative rosetting receptor, CR1 [9], is commonly deficient in the PNG population [46]. CR1-binding IE may not be able to sequester efficiently, possibly preventing a severe infection from being established and antibodies to be generated against them. While 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 [47].

Impaired consciousness, respiratory distress and severe anaemia are the commonly overlapping severe malaria syndromes in young African children. Of these, impaired consciousness and respiratory distress are the strongest predictors for mortality [48]. In these studies, respiratory distress (acidotic breathing) was considered a surrogate for metabolic acidosis, through increased respiratory drive. In our study, we found that in convalescence, younger children with metabolic acidosis had higher antibody levels against rosetting-associated CIDRδ domains than children without metabolic acidosis. In Kenyan children with severe malaria [49], the rosetting frequency of the infecting parasites correlated with the degree of metabolic acidosis. This suggests that PfEMP1s containing CIDRδ domains may be associated with metabolic acidosis, with rosette formation as a possible link [49]. In the same study, it was shown that expression of Group A-like *var* genes and absence of rosetting was associated with impaired consciousness [49]. In the present study we also found that children with impaired consciousness had higher convalescent antibody levels 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 analysed 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 [50]. Studies of antibody acquisition to defined antigens associated with malaria only provide 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 which limited our ability to investigate how antibody responses differed between severe malaria syndromes. Further experiments validating 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]) are required. Studies investigating the blocking of the CIDRα1-EPCR binding by these antibodies would further corroborate the findings here.

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 if these antibodies confer protection against severe malaria.

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Footnote

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Conflict of interests

No conflicts of interest reported by the authors

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Figure legends

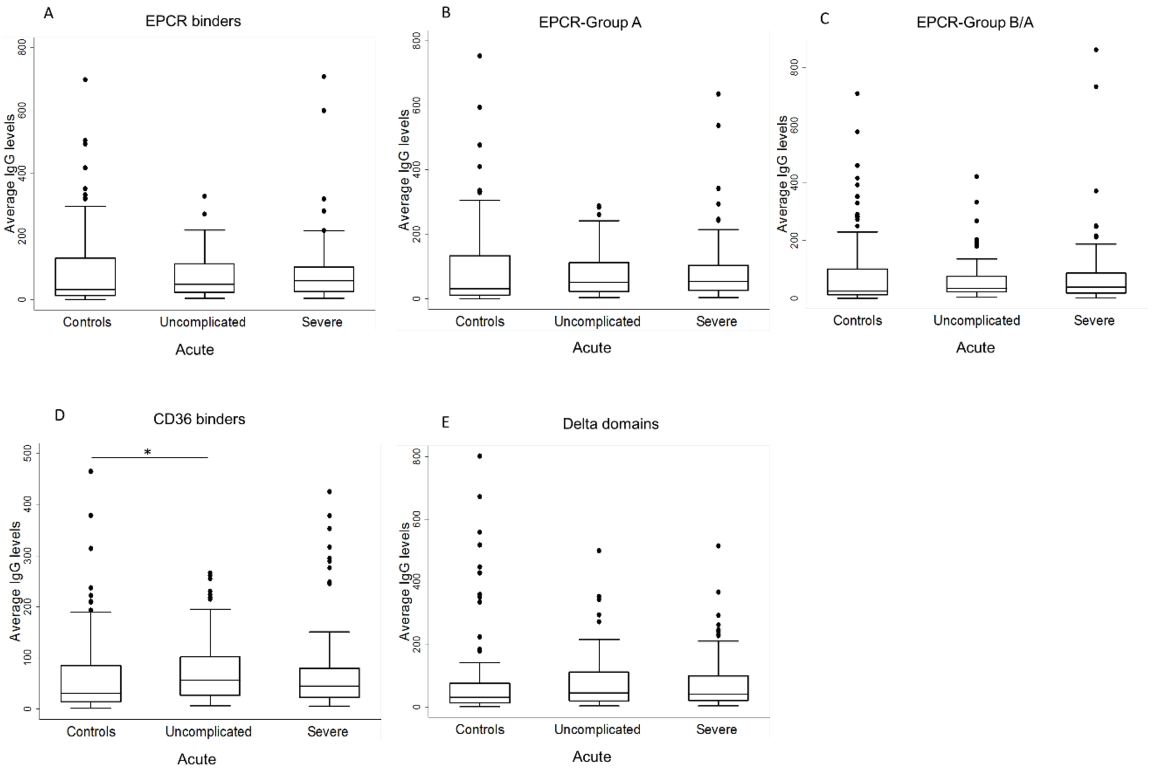

Figure 1. IgG levels against different CIDR domain types in participants belonging to different disease severity groups at baseline (acute). Using Kruskal Wallis test, the community controls, participants with uncomplicated acute malaria and severe acute malaria were compared. Y-axes represent average IgG levels in Relative Units. Box plot shows median with lower 25th percentile and upper 75th percentile and outliers. Total number of participants in each category: community control, n=112; uncomplicated acute, n=65 severe acute, n=98. 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, * p=0.0109, Wilcoxon Rank Sum test. E: Delta domains associated with rosetting. IgG, immunoglobulin G; CIDR, cysteine-rich inter-domain region; EPCR, endothelial protein C receptor; CD36, cluster of differentiation 36.

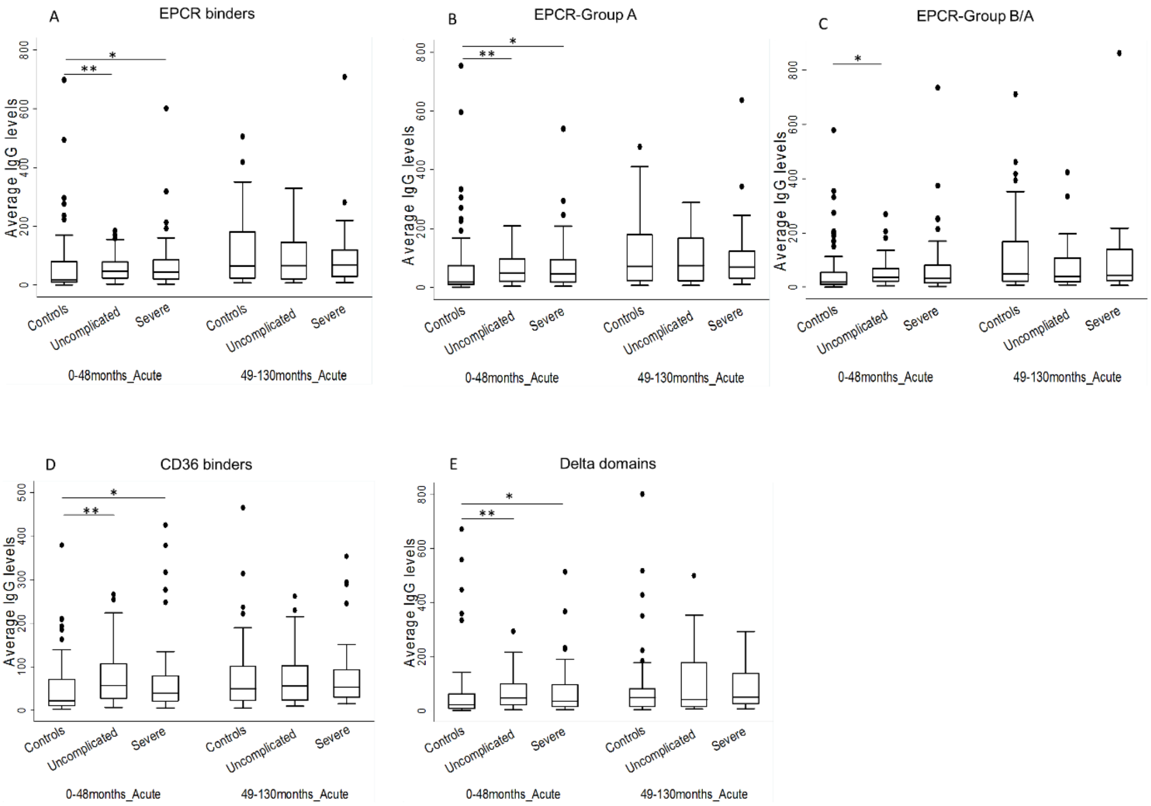

Figure 2. **IgG levels against different CIDR domain types in participants belonging to different disease severity groups and grouped according to age at baseline (acute)**. Using Wilcoxon Rank Sum test, participants in the community controls, uncomplicated acute malaria and severe acute malaria group in the 0-48 months age group were compared. Similar comparisons were done for participants in 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 community controls group, uncomplicated acute malaria group and severe acute 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=0.019; ** p=0.0199. B: Binding EPCR and belonging to Group A, * p=0.0268; ** p=0.0332. C: Binding EPCR and belonging to Group B/A, * p=0.0184.

D: Binding CD36, * p=0.034; ** p=0.0016. E: Delta domains associated with rosetting, * p=0.0271; ** p=0.0188. IgG, immunoglobulin G; CIDR, cysteine-rich inter-domain region; EPCR, endothelial protein C receptor; CD36, cluster of differentiation 36.

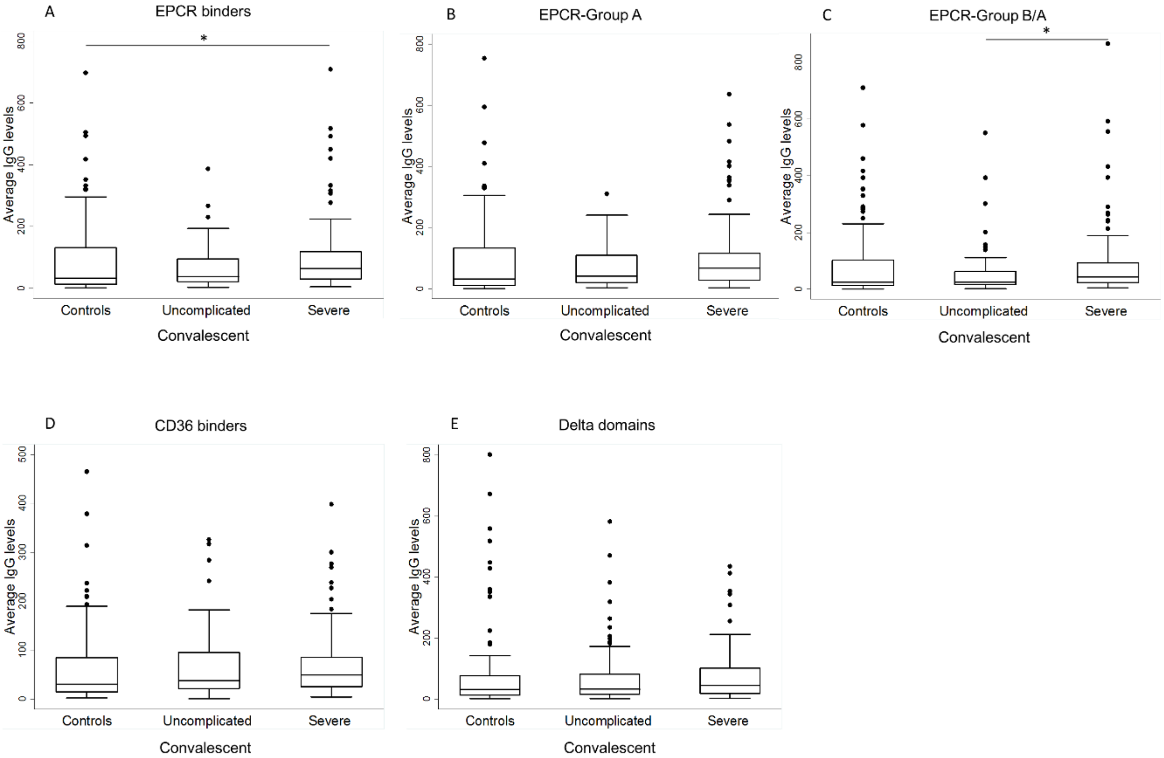

Figure 3. IgG levels against different CIDR domain types in convalescent plasma from children belonging to different disease severity groups. Using Kruskal Wallis test, community controls, and convalescent plasma from uncomplicated malaria and severe malaria groups were compared. Y-axes represent average IgG levels in Relative Units. Box plot shows median with lower 25th percentile and upper 75th percentile and outliers. Total number of participants in each category: community controls, n=112; uncomplicated convalescent, n=65; severe convalescent, n=98. Average IgG levels against the following CIDR domain types were measured: A: Binding EPCR, * $p=0.0324$, Wilcoxon Rank Sum test. B: Binding EPCR and belonging to Group A. C: Binding EPCR and belonging to Group B/A, * $p=0.0125$, Wilcoxon Rank Sum test. D: Binding CD36. E: Delta domains associated with rosetting. IgG, immunoglobulin G; CIDR, cysteine-rich inter-domain region; EPCR, endothelial protein C receptor; CD36, cluster of differentiation 36.

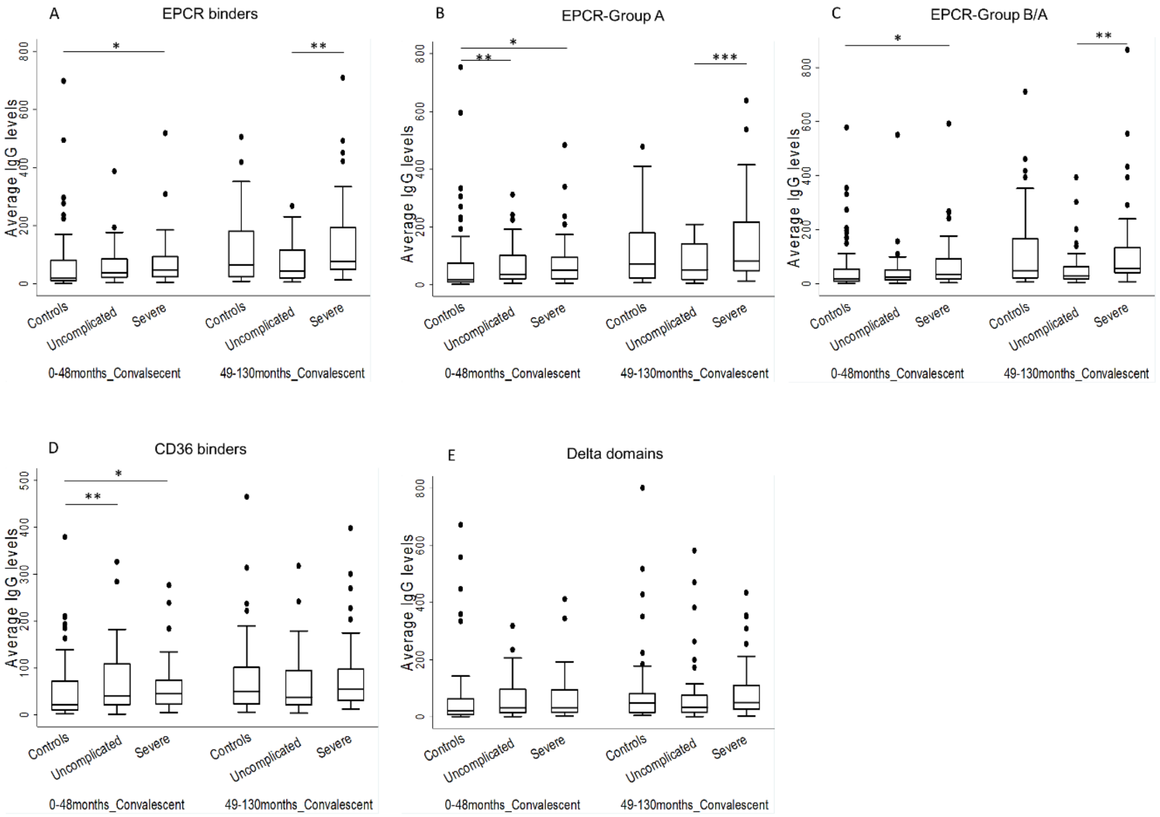

Figure 4. IgG levels against different CIDR domain types in convalescent plasma from children belonging to different disease severity groups and grouped according to age. Using Wilcoxon Rank Sum test, community controls, convalescent plasma from uncomplicated malaria and severe malaria in the 0-48 months age group were compared. Similar comparisons were done for participants in 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 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=0.0122, ** p=0.023. B: Binding EPCR and belonging to Group A, * p=0.0106; ** p=0.0411, *** p=0.0369. C: Binding EPCR and belonging

to Group B/A, * p=0.0344; ** p=0.0177. D: Binding CD36, * p=0.0477; ** p=0.0.0481. E: Delta domains associated with rosetting. IgG, immunoglobulin G; CIDR, cysteine-rich inter-domain region; EPCR, endothelial protein C receptor; CD36, cluster of differentiation 36.

Tables

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 (months)[b]	42 [30-60]	45 [34-59]	42 [30-58]
Age group[c]			
0-48 (months)	61.2	53.9	57.7
49-130(months)	38.8	46.1	42.3
Sex[c]			
(Female)	45.9	41.5	
(Male)	54.1	58.5	

Ethnicity[c]			
(Madang)	78.6	89.2	79.5
(Madang Sepik)	7.1	4.6	9.8
(Other)	5.1	6.2	
(Sepik)	9.2		10.7
Haemoglobin (g/L) [b]	79 [55-91]	89 [72-100]	104 [92-113]
Initial P. falciparum density (parasites/µl) [b]	86171.5 [27064-181072]	21938 [4811-66053]	

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

[b] Data provided for age, haemoglobin and initial *P. falciparum* density are the median values and [interquartile range]

[c] Age groups, sex and ethnicity are represented by percentages

Table 2. Change in IgG levels over time in the severe malaria group against different CIDR domain types (Severe Convalescent Vs Severe acute[a]).

CIDR domain type	**Severe malaria**		
	Median [IQR]		**P-value[b]**
	Acute	**Conv.**	
CIDR EPCR	60.5 [25.3-102.8]	63.8 [29.1-118.4]	0.039
CIDR EPCR Group A	53.4 [25.5-106.0]	67.2 [26.9-116.9]	0.018
CIDR EPCR Group B/A	39.4 [18.5-94.9]	43.7 [21.2-93.6]	0.130
CIDR CD36	44.9 [22.7-79.9]	49.5 [25.7-86.2]	0.949
CIDR delta	41.1 [20.4-102.6]	44.1 [18.5-101.5]	0.771

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 severe malaria, n=98.

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

594 **Table 3.** Change in IgG levels over time in the severe malaria group against different CIDR domain types in participants grouped by age (Severe

595 convalescent Vs Severe acute[a]).

CIDR domain type	**Severe malaria**			
		Median [IQR]		**P-value[b]**
	Age group (months)	**Acute**	**Conv.**	
CIDR EPCR	0-48	44.6 [19.6-89.3]	47.1 [21.7-93.2]	0.529
	49-130	68.3 [28.7-121.5]	75.8 [47.3-199.3]	0.015
CIDR EPCR Group A	0-48	46.1 [17.6-95.5]	49.3 [19.5-94.2]	0.391
	49-130	68.3 [29.2-123.6]	81.4 [45.9-223.3]	0.013
CIDR EPCR Group B/A	0-48	31.6 [13.8-81.3]	34.6 [15.6-91.9]	0.811

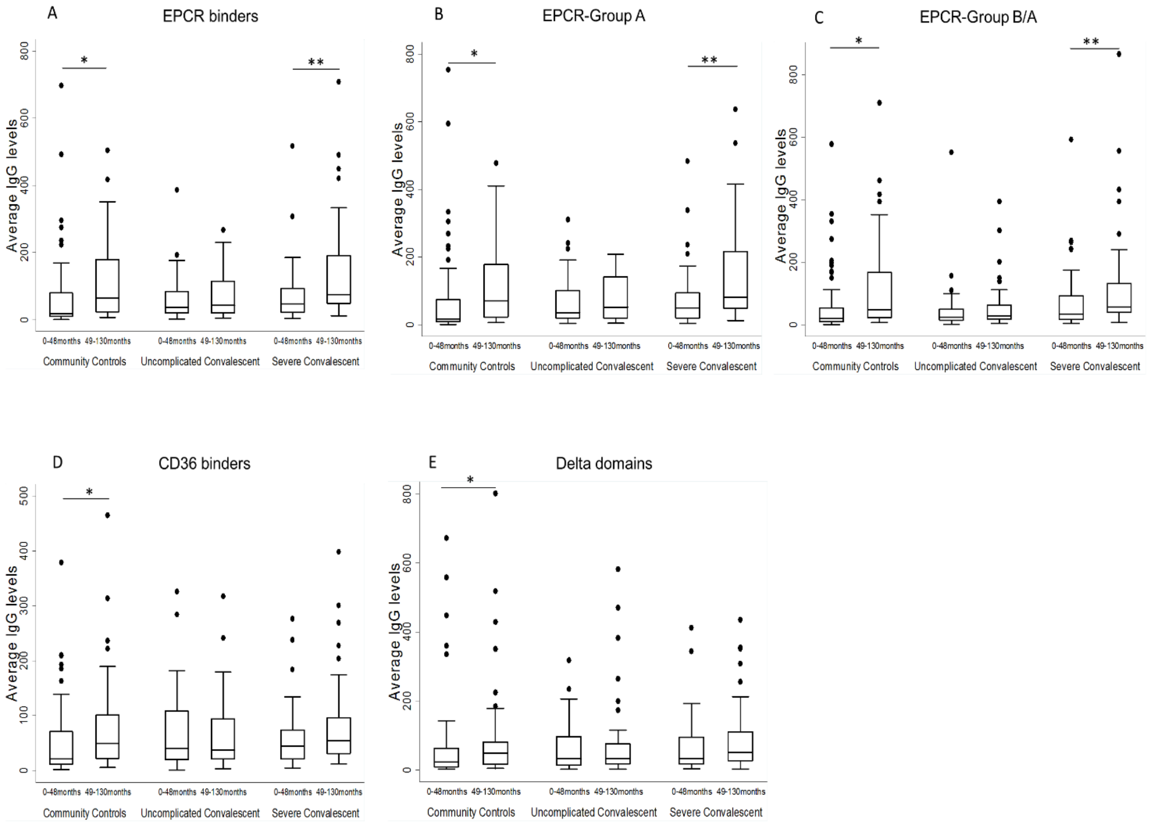
	49-130	43.5 [24.5-140.2]	56.1 [38.2-145.6]	0.036
CIDR CD36	0-48	38.7 [20.5-79.2]	44.7 [21.5-74.7]	0.558
	49-130	53.0 [29.5-93.1]	54.5 [29.5-104.7]	0.602
CIDR delta	0-48	35.1 [16.3-97.6]	32.4 [15.9-95.6]	0.464
	49-130	50.5 [25.8-139.5]	50.8 [26.6-113.3]	0.612

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

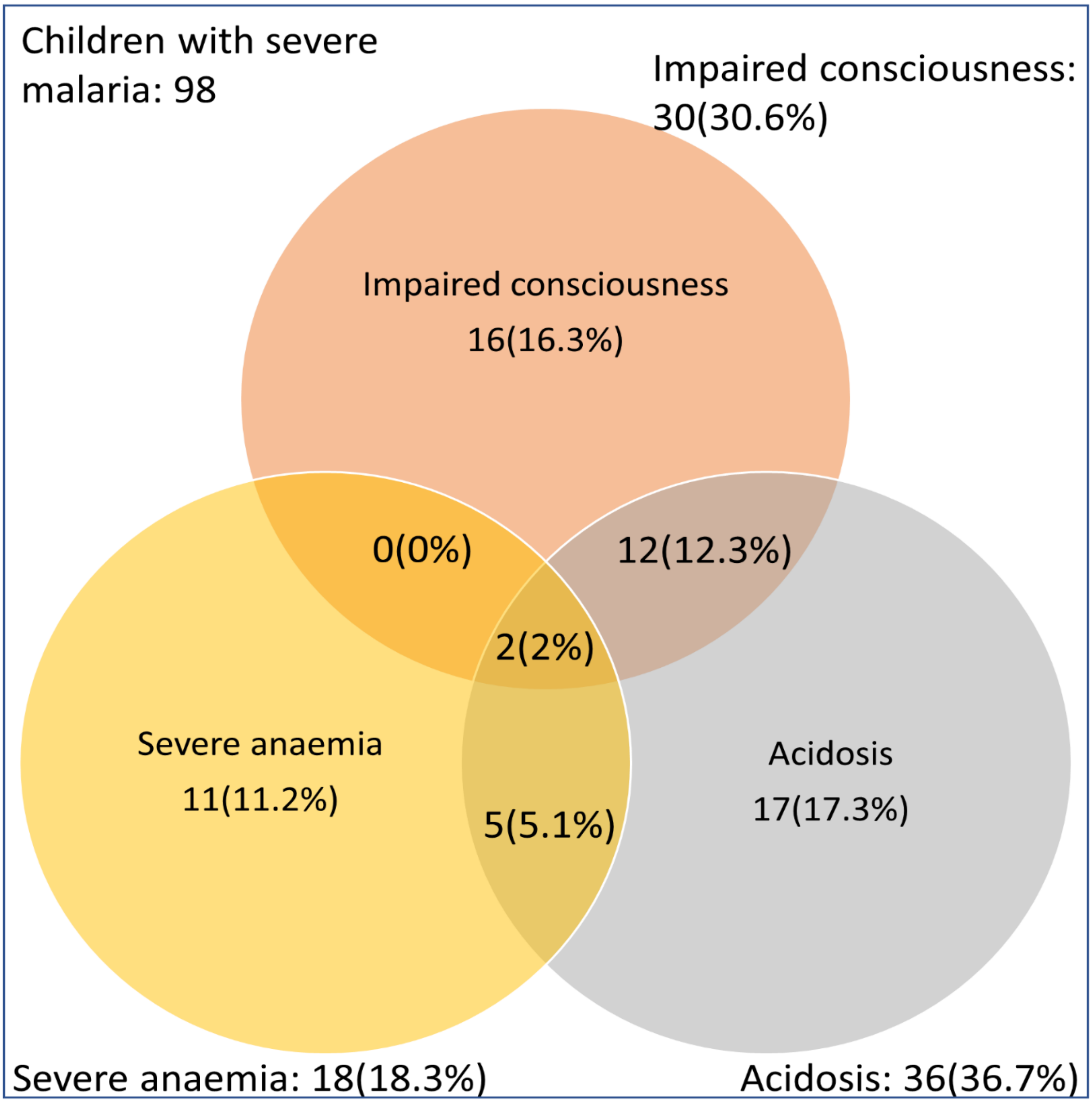
[a] Total number of participants with severe malaria in 0-48 months age group: n=60 and in the 49-130 months age group: n=38.

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

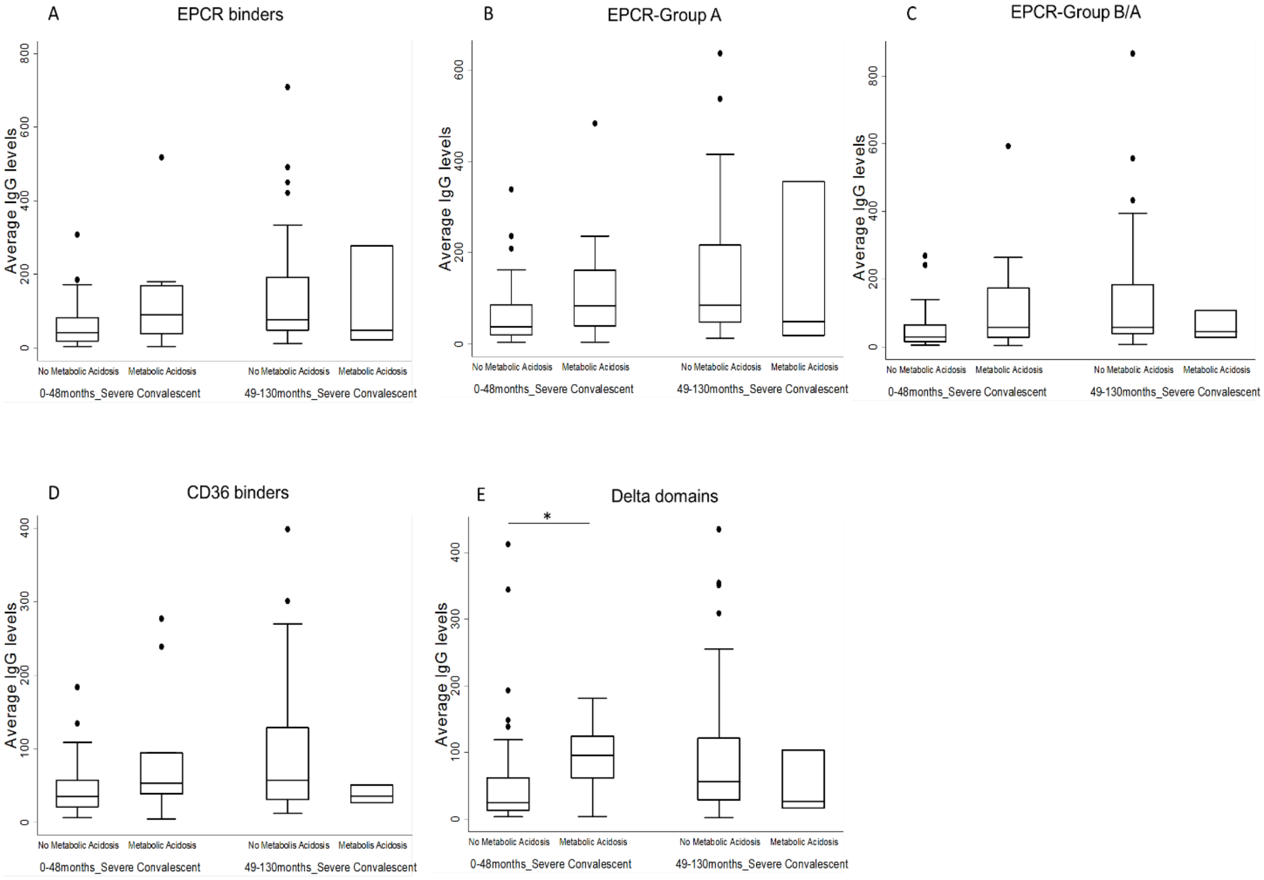
Supplementary figures



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.

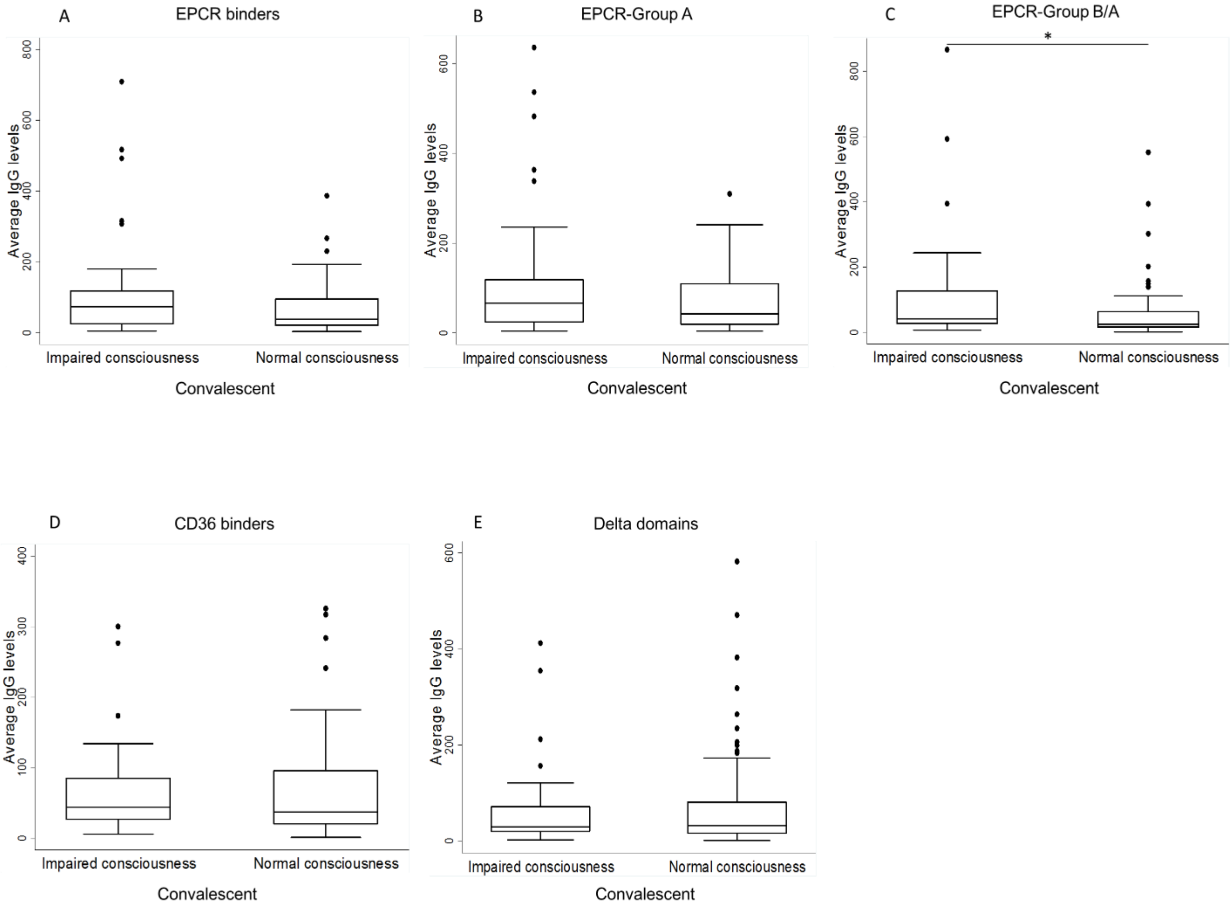


Supplementary Figure 2. Overlapping severe malaria syndromes in children with severe malaria. The overlapping severe malaria syndromes in children with severe malaria (n=98) have been described using a Venn diagram. Children with severe anaemia (n= 18), impaired consciousness (blantyre coma score, BCS 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 [1, 5]	α1.7 (b)	α1.7		A	EPCR	1918	1918_3	CIDR EPCR and Group A 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 [2, 6]	α2.9	α2.9		B	CD36	IT4	IT4var45	CIDR CD36
CIDRα3.1_DD2var01_DD2 [2, 6]	α3.1 (a)	α3.1		B	CD36	DD2	DD2var01	CIDR CD36
CIDRα3.1_HB3var27_HB3	α3.1 (b)	α3.1		B	CD36	HB3	HB3var27	CIDR CD36

[2, 7]							
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	δ (c)	δ	A	unknown	IT4	IT4var02	CIDR delta

[2, 7]							
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

PICGVKCENKSCTDKENDDDCKNKKKYDPPKGVTPIDIPILYSGDKQGDITKKLEDFCYNRTKENEKTYQNWKCYYKDSEFNKCKMESKSGKSTTQEKIISFDEFFYLWVNNLLIDSIMWENDIKHCINNTNVTNCKNKCNENCKCFKNWVKKKEEEWTKVKQILGNRSENLNNYYNKLNSLFKGFFFEVVYKFNNKEEKWNKLTEKLEQKIGSSKGKEGVENPKDAIELLLDHLKENAITCKDNNSLEEDKNCPKIKINPC

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

PYCGMKKKGDNWVAKENDENCKRGNLYTILTNAESTNIDVLSFGDKREDRETKLIKFCAEKNGGVAGGGGSGSNSNSKELYEEWKCYKHDYVKEVGEKDEDEEENLEKVKAAGGLCILKKEKKGVEETNSQKEPDEIQKTFNPFFYYWVAHMLKDSIYWETQKIKKCLKKGTKIKCTEKCKRDCGCFKRWVEQKQTEWKAIKKHFKTQDNIIEGFHDITLKEVLKLEFENKNTEEDKENNVSAEEIDLINKMLKEDETAVAGASGGEDNTTIDKLLKHELDEAKQCIKKC

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 CIDR domains	**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.

References for supplementary information

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