Antibody targets on the surface of *P. falciparum*-infected erythrocytes that are associated with immunity to severe malaria in young children

Authors/Affiliations:

Jo-Anne Chan[1], Michelle J Boyle[1], Kerryn A Moore[1,3,4], Linda Reiling[1], Zaw Lin[1], Wina Hasang[2], Marion Avril[8], Laurens Manning[5,7], Ivo Mueller[6], Moses Laman[5], Timothy Davis[7], Joseph D Smith[8], Stephen J Rogerson[2], Julie A Simpson[3], Freya JI Fowkes[1,3,9], James G Beeson[1,2,10]

[1]Burnet Institute for Medical Research and Public Health, Melbourne, Australia

[2]Department of Medicine and [3]Centre for Epidemiology and Biostatistics, Melbourne School of Population and Global Health, University of Melbourne, Parkville, Australia

[4]Murdoch Children's Research Institute, Royal Children's Hospital, Melbourne, Australia

[5]Papua New Guinea Institute of Medical Research, Madang, Papua New Guinea

[6]Walter and Eliza Hall Institute of Medical Research, Parkville, Australia

[7]University of Western Australia, Perth, Australia

[8]Center for Infectious Diseases Research, Seattle, Washington, USA

[9]Department of Epidemiology and Preventive Medicine and Department of Infectious Diseases, [10]Department of Microbiology, Monash University, Melbourne, Victoria, Australia

ABSTRACT

Background

Sequestration of *P. falciparum*-infected erythrocytes (IEs) in the microvasculature contributes to pathogenesis of severe malaria in children. This mechanism is mediated by antigens expressed on the IE surface. However, knowledge of specific targets and functions of antibodies to IE surface antigens that protect against severe malaria is limited.

Methods

Antibodies to IE surface antigens were examined in a case-control study of young children in Papua New Guinea presenting with severe or uncomplicated malaria (n=448), using isolates with a virulent phenotype associated with severe malaria, and functional opsonic phagocytosis assays. We used genetically-modified isolates and recombinant PfEMP1 domains to quantify PfEMP1 as a target of antibodies associated with disease severity.

Results

Antibodies to the IE surface and recombinant PfEMP1 domains were significantly higher in uncomplicated versus severe malaria and were boosted following infection. Using genetically-modified *P. falciparum* revealed that PfEMP1 was a major target of antibodies and PfEMP1-specific antibodies were associated with reduced odds of severe malaria. Furthermore, antibodies promoting the opsonic phagocytosis of IEs by monocytes were lower in those with severe malaria.

Conclusion

Findings suggest PfEMP1 is a dominant target of antibodies associated with reduced risk of severe malaria, and function in part by promoting opsonic phagocytosis.

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INTRODUCTION

The global burden of malaria has declined in recent years due to improved access to malaria interventions [1]. However, challenges of resistance to anti-malarial drugs have escalated the need for an effective vaccine. The most advanced vaccine, RTS,S, has only ~30% efficacy in children [2]. To develop malaria vaccines with increased efficacy, especially against severe malaria, further understanding of the targets of antibody responses that protect against disease is required. Among endemic populations with a high transmission levels, severe malaria mainly affects young children [3]. The pathogenesis of severe malaria from *Plasmodium falciparum*, the major cause of human malaria, is in part due to the sequestration of large numbers of mature *P. falciparum*-infected erythrocytes (IEs) in the microvasculature of specific organs (reviewed in [4]). The mechanical obstruction of blood flow and associated inflammation contribute to the manifestation of severe disease complications such as cerebral malaria [5-8].

Sequestration is mediated by the specific interaction of PfEMP1, expressed on the IE surface, with receptors on the host endothelium (reviewed in [4]). PfEMP1 is encoded by the *var* multigene family [9], which can be divided into three main groups (A, B, C) and a chimeric group B/A *var* gene (termed DC8) based on their upstream promoter regions [10]. Transcription of different *var* gene subgroups has been linked to clinical disease manifestations [11]. Expression of group A *var* genes has been associated with severe malaria in children from Tanzania and Papua New Guinea (PNG) [12-14]. Group A and

B *var* genes encode PfEMP1 variants involved in key pathogenic features of severe malaria, such as rosetting [15,16] and adhesion to ICAM-1 on brain endothelium [17]. Despite the high rate of *var* gene recombination, certain tandem domain arrangements of the extracellular portion of PfEMP1, also known as domain cassettes (DCs), appear to be highly conserved. A subset of Group A *var* genes and the DC8 *var* gene can bind to endothelial protein C receptor (EPCR) expressed by human brain endothelial cells [18], contributing to the pathogenesis of severe malaria [19]. Severe malaria in children was associated with expression of PfEMP1 variants containing DC8 (Group B/A) and DC13 (group A) domain arrangements [20-22], which bind to EPCR [18,23,24]. DC13 PfEMP1 has dual specificity and adheres to EPCR and ICAM-1 on brain endothelial cells [25,26]. Parasites from cerebral malaria patients were also more likely to bind EPCR and ICAM-1 than those with uncomplicated malaria [19]. Other parasite proteins identified on the IE surface have also been proposed to play roles in disease pathogenesis, including RIFIN, STEVOR and SURFIN [27-31].

After repeated exposure to *P. falciparum*, individuals living in malaria-endemic regions can acquire immunity that protects against severe disease [32-34]. However, targets and mechanisms of immunity to severe malaria are poorly understood. PfEMP1 and other IE surface antigens have been identified as key targets of acquired antibodies (reviewed in [4]). Prior studies using genetically-modified *P. falciparum* with suppressed PfEMP1 expression, and other approaches, demonstrated that PfEMP1 is a dominant IE surface target of naturally-acquired antibodies and found that PfEMP1-specific antibodies were associated with protection against uncomplicated pediatric malaria [35-37]. Some studies have found associations between antibodies to recombinant PfEMP1 domains and protection from uncomplicated malaria, although findings have not been highly consistent (reviewed in [4]).

Much less is known about responses mediating protection from severe malaria. Studies have suggested that young children tend to first acquire antibodies to PfEMP1 encoded by group A and DC8 *var* genes, that are associated with severe disease [12,38], compared to groups B and C; this may contribute to protection from severe disease [39,40]. In several small studies, it was reported that children with severe malaria had antibodies that recognized DC8 and DC13 PfEMP1 variants [20-22]. Antibodies to IEs can promote opsonic phagocytosis by monocytes. This is thought to play a major role in immunity, but the contribution of opsonic phagocytosis to immunity against severe malaria has not been investigated. Limited data is available on the association between antibodies to PfEMP1 and protection against severe malaria or quantifying PfEMP1 and other IE surface antigens as antibody targets on IEs during severe malaria. Currently, very little is known regarding immunity to severe malaria in non-African populations.

In the present study, we evaluated the acquisition of naturally-acquired antibodies to IE surface antigens in a case-control study of children (n=448) in Papua New Guinea (PNG), presenting with severe or uncomplicated malaria. We studied the importance of PfEMP1 and other IE surface antigens as targets of naturally-acquired antibodies and related these to protective associations. We compared antibody responses between severe and uncomplicated malaria, during acute infection and following convalescence, to evaluate the acquisition of immunity. We used *P. falciparum* isolates expressing PfEMP1 variants associated with severe malaria to quantify the levels of acquired antibodies. We investigated the significance of PfEMP1 as an antibody target using genetically-modified *P. falciparum* with substantially reduced PfEMP1 expression and using recombinant PfEMP1 domains. Additionally, we evaluated the functional

importance of acquired antibodies in their ability to mediate the opsonic phagocytosis of IEs.

METHODS

A comprehensive description of the methods used in this study is in Supplementary Materials.

Study population

Samples for antibody measurement were extracted for a frequency-matched case-control study of children presenting with severe or uncomplicated malaria in Madang, Papua New Guinea from 2006 to 2009 [41]. This case-control study was nested within a cohort study described elsewhere [41]. Blood samples were collected from children (n=805; age range 2 months-10 years; Supplemental Table S1) at enrolment (acute infection) and 2 months post-infection (convalescence). A summary of demographic and malariometric characteristics of children presenting with uncomplicated and severe malaria is presented in Table 1.

Ethics statement

Ethics approval was obtained from the PNG Medical Research Advisory Committee, PNGIMR Institutional Review Board, and Alfred Hospital HREC. Written informed consent was obtained from all study participants or their legal guardians.

P. falciparum culture and isolates

P. falciparum isolates were maintained in continuous culture and synchronized as previously described [35,36]. 3D7vpkd [35,42] and 1E2 (IT4var19) parasites [20] were generated as previously described.

153

154 **Measuring antibodies to the IE surface by flow cytometry**

155 Measuring IgG binding to the IE surface of pigmented-trophozoites was performed with
156 an established flow cytometry-based assay, as described [35].

157

158 **Measuring antibodies to recombinant PfEMP1 domains by ELISA**

159 Antibodies to recombinant domains of PfEMP1 (DBLα2, CIDRα1, DBLβ12 and DBLγ6)
160 was measured by ELISA using established methods [43].

161

162 **Measuring opsonic phagocytosis**

163 The level of opsonic phagocytosis to the IE surface was measured by flow cytometry, as
164 described [35,44].

165

166 **Statistical analyses**

167 For the primary analyses, multivariable logistic regression models were used to estimate
168 the associations between total antibody responses and severe *P. falciparum* malaria,
169 adjusting for age, sex, and ethnicity.

170

171 **RESULTS**

172

173 **PfEMP1 is a dominant target of naturally-acquired antibodies to the IE surface**
174 **among young children with severe or uncomplicated malaria**

175 To quantify the role of PfEMP1 as a target of acquired antibodies, we measured
176 antibody reactivity to the IE surface using an established flow cytometry-based assay
177 [35]. We used a 3D7 isolate predominantly expressing PfEMP1 variants associated with
178 virulent phenotypes that contribute to severe malaria [37]. The dominant PfEMP1

179 expressed by our 3D7 isolate is a group A type (PF11_0521) with a DC13 domain
180 structure that mediates adhesion to endothelial cells [37]. Two other group A *var* genes
181 were also upregulated (PFD1235w and PFA0015c) [37]. We compared antibody levels
182 of 3D7parental to a transgenic line with inhibited PfEMP1 surface expression due to
183 endogenous *var* gene suppression (*var* promoter 'knock-down'; 3D7vpkd) [35,42]; this
184 isolate has greatly reduced PfEMP1 expression, but still expresses other antigens,
185 including RIFIN and STEVOR [35]. We measured the level of IgG binding to the IE
186 surface in serum samples collected at enrolment (acute infection) from children with
187 severe (SM; n=235) or uncomplicated malaria (UM; n=213). Antibody levels were further
188 measured in serum samples collected 2 months post-infection (following convalescence)
189 from the same children (SM, n=184; UM, n=173). All individuals at both time-points
190 showed a marked reduction in IgG binding to 3D7vpkd compared to 3D7parental (Fig 1A,
191 1C). Overall, during acute infection, IgG binding to 3D7vpkd was substantially reduced
192 by 41.7% and 59.5% compared to 3D7parental, for SM and UM respectively (Fig 1B,
193 $p<0.001$; Supplemental Fig S1A, S2). Similarly, at convalescence, IgG binding to
194 3D7vpkd was reduced by 48.6% and 67.2% compared to 3D7parental for SM and UM
195 respectively (Fig 1D, $p<0.001$; Supplemental Fig S1B). While the antibody reactivity to
196 3D7vpkd was greatly reduced compared to 3D7parental, in both SM and UM, there was
197 a strong, positive correlation between antibody responses to 3D7parental and 3D7vpkd
198 at acute infection (SM, Spearman rank correlation coefficient, r_s 0.54, $p<0.0001$; UM, r_s
199 0.68, $p<0.0001$) and following convalescence (SM, r_s 0.56, $p<0.0001$; UM, r_s 0.65,
200 $p<0.0001$). Our findings suggest that PfEMP1 is a major target of naturally-acquired
201 antibodies, consistent with our previous reports (which did not include individuals with
202 severe malaria) [35,36].
203
204 **Antibodies to the IE surface are higher in uncomplicated malaria**

Children with SM and UM had similar age, sex and ethnicity (characteristics used for matching), but SM children had significantly higher parasitemia, which is a common feature of SM (Table 1). IgG binding to the IE surface of 3D7parental was 57.5% and 64.3% higher in UM compared to SM during acute infection (Fig 2A; $p<0.0001$) and following convalescence (Fig 2B; $p=0.0002$). In contrast, there was a trend of lower IgG binding to 3D7vpkd in UM compared to SM samples, at acute infection (Fig 2A; $p=0.08$) and following convalescence (Fig 2B; $p=0.06$). Children with antibodies to 3D7parental and PfEMP1-specific antibodies (calculated as IgG levels to 3D7parental minus 3D7vpkd) had reduced odds of SM relative to UM, but this was not observed for those with antibodies to 3D7vpkd (Supplemental Table S2). We also measured antibody levels towards the IE surface of a genetically different isolate, 1E2 (IT4var19) (20), which expresses a specific PfEMP1 variant (DC8-type) with a virulent phenotype. Similar to 3D7parental, IgG binding to 1E2 (IT4var19) was higher in UM compared to SM, during acute infection (Fig 2C; 28.6% higher, $p<0.0001$) and following convalescence (Fig 2D; 26.4% higher, $p=0.0008$). Children with antibodies to 1E2 (IT4var19) parasites had reduced odds of SM relative to UM (Supplemental Table S2).

Antibodies to the IE surface are boosted by infection

Next, we compared antibody responses within individuals using samples that were collected at acute infection and at convalescence to determine antibody boosting. IgG binding to 3D7parental and 3D7vpkd was higher following convalescence for both SM (Fig 3A; 18.4% higher for 3D7parental, 3.84% higher for 3D7vpkd, $p<0.001$; Supplemental Fig S1C) and UM (Fig 3B; 31.4% higher, $p=0.002$ for 3D7parental, 38.4% higher, $p=0.04$ for 3D7vpkd; Supplemental Fig S1D). IgG binding to 1E2 (IT4var19) parasites was also slightly higher during convalescence for SM (Fig 3C; 6.2% higher, $p<0.0001$) and UM (Fig 3D; 3.3% higher, $p=0.1$). Comparing the magnitude of antibody

231 boosting in children (calculated as antibody levels at convalescence minus acute), there
232 was an increase in the magnitude of antibody levels observed for SM and UM, for both
233 3D7parental and 3D7vpkd parasites (Supplemental Fig S3A). Antibody boosting to 1E2
234 (IT4var19) parasites was only observed with SM (Supplemental Fig S3B). Greater
235 boosting of antibodies to 3D7 may indicate that this isolate expresses antibody epitopes
236 or antigenic determinants that are more common in our study population than those
237 expressed by 1E2.
238
239 No significant correlation was observed between antibodies to 3D7parental and
240 3D7vpkd for SM and UM, at acute and convalescence (Supplemental Table S3).
241 However, there was a moderate, positive correlation between antibodies to 1E2
242 (IT4var19) parasites for SM and UM, at acute and convalescence (Supplemental Table
243 S3). There was a weak, positive correlation between antibodies to 3D7parental and 1E2
244 (IT4var19) for SM measured at acute infection (Supplemental Table S4), but no
245 correlation was observed for SM at convalescence. Similarly, no correlation was
246 observed for antibodies to 3D7vpkd and 1E2 (IT4var19) parasites for SM at acute or
247 convalescence (Supplemental Table S4). In UM, there was a strong, positive correlation
248 between antibodies to 3D7parental and 1E2 (IT4var19), and moderate positive
249 correlation between 3D7vpkd and 1E2 (IT4var19), at acute and convalescence
250 (Supplemental Table S4).
251
252 **Antibodies to specific recombinant 1E2 (IT4var19) PfEMP1 domains are higher in**
253 **uncomplicated malaria**
254 We tested serum samples for antibodies to four recombinant domains of the PfEMP1
255 variant encoded by 1E2 (IT4var19) parasites (DBLα2, CIDRα1, DBLβ12 and DBLγ6) to
256 further evaluate the significance of PfEMP1 as a target of acquired antibodies and

examine whether responses to specific PfEMP1 domains may be important in protection from severe disease. During acute infection, the level of IgG binding was significantly higher in UM compared to SM for DBLα2 (Fig 4A; 17.4% higher, *p*=0.04) and DBLγ6 (Fig 4D; 17.6% higher, *p*=0.006). This was not observed for CIDRα1 (Fig 4B; 7.6% lower, *p*=0.28) and DBLβ12 (Fig 4C; 3.2% higher, *p*=0.39). Children with antibodies to DBLα2 and DBLγ6 had reduced odds of SM relative to UM (Supplemental Table S5). Antibodies to recombinant PfEMP1 domains were correlated, suggesting co-acquisition (Supplemental Table S6). There was a strong, positive correlation observed between antibody responses to 1E2 (IT4var19) parasites and the recombinant PfEMP1 domains DBLα2 and DBLγ6, but not CIDRα1 or DBLβ12, at acute infection for SM and UM (Supplemental Table S7).

Antibodies that mediate the opsonic phagocytosis of IEs are higher in uncomplicated malaria and target PfEMP1

To quantify the functional capacity of antibodies targeting IE surface antigens, SM and UM samples at acute infection were tested in an established opsonic phagocytosis assay using undifferentiated THP-1 monocytes [35,44]. The majority of individuals showed a marked reduction in phagocytosis activity with 3D7vpkd compared to 3D7parental (Fig 5A). Overall, the level opsonic phagocytosis activity was markedly reduced in 3D7vpkd for both SM and UM samples (Fig 5B; *p*<0.001), indicating that PfEMP1 is a major target of functional antibodies that promote IE phagocytosis. Opsonic phagocytosis activity was higher in UM compared to SM for both 3D7parental (Fig 5C; 24% higher, *p*=0.03) and 3D7vpkd (Fig 5C; 27% higher, *p*<0.001). Children with antibodies that promote opsonic phagocytosis of 3D7parental, 3D7vpkd and 3D7-PfEMP1 had reduced odds of SM relative to UM (Supplemental Table S2). The level of total IgG binding to the IE surface and opsonic phagocytosis activity was not directly

283 correlated for SM or UM samples for 3D7parental and 3D7vpkd (Supplemental Fig S4),
284 suggesting that total IgG levels may not be a good measure of antibody function.
285
286 **DISCUSSION**
287 In this study we found that children with uncomplicated malaria had significantly higher
288 antibodies to IE surface antigens and PfEMP1 specifically. We demonstrated this by
289 quantifying antibodies with two different IE isolates that express virulent PfEMP1 types
290 associated with severe malaria pathogenesis, alongside genetically-modified *P.*
291 *falciparum* with suppressed PfEMP1 expression and using recombinant PfEMP1
292 domains. We demonstrated that PfEMP1 is a major target of naturally-acquired
293 antibodies to the IE surface in these children; importantly, PfEMP1-specific antibodies,
294 quantified using native proteins expressed on the IE surface and recombinant antigens,
295 were higher in those with uncomplicated malaria, and associated with reduced odds of
296 SM. This suggests that PfEMP1-specific antibodies play a role in protection from severe
297 malaria. Furthermore, antibodies to IE surface antigens were boosted following either
298 severe or uncomplicated malaria. Antibodies promoting opsonic phagocytosis were
299 higher in children with UM and associated with protection from SM, suggesting functional
300 antibodies have important roles in immunity from severe malaria. Together, our results
301 suggest the importance of antibodies to the IE surface, predominantly PfEMP1, in
302 contributing to protective immunity against severe malaria in young children.
303
304 The overall level of IgG binding to the IE surface was higher in children with
305 uncomplicated malaria, compared to severe malaria, for IEs of 3D7 and 1E2 (IT4var19).
306 This difference was observed in samples collected at acute infection and following
307 convalescence. The 3D7 and 1E2 (IT4var19) isolates were used because they are
308 known to express virulent PfEMP1 types associated with severe malaria pathogenesis.

309 The transcription level of both group A and DC8 EPCR-binding *var* genes is increased in
310 severe malaria infections [18,22,45-47]. The dominant PfEMP1 expressed by our 3D7
311 isolate is a group A type (PF11_0521) that has a DC13 domain structure that mediates
312 adhesion to endothelial cells [37], and two other group A *var* genes were also
313 upregulated (PFD1235w and PFA0015c) [37]. The 1E2 (IT4var19) parasite line
314 expresses a specific DC8 PfEMP1 that was upregulated when parasites were selected
315 for adhesion to brain endothelial cells [20]. The PfEMP1 variant expressed by the 1E2
316 (IT4var19) parasites is a DC8 arrangement associated with severe disease. Our findings
317 suggest that higher levels of antibodies to group A and DC8 PfEMP1 contribute to
318 protection from severe malaria. Future studies in additional populations might enable the
319 identification of antibody thresholds for protection against severe malaria. In our studies
320 we considered all children meeting the criteria of severe malaria in one group and did
321 not perform analyses of sub-groups different severe malaria syndromes, which could be
322 considered in future studies.
323
324 Comparing antibody responses between 3D7parental and 3D7vpkd IEs allowed
325 quantification of PfEMP1-specific antibodies. Overall IgG binding to the IE surface of
326 3D7vpkd was markedly reduced compared to 3D7parental, indicating that the majority of
327 acquired antibodies to the IE surface are targeting PfEMP1. The decrease in IgG binding
328 to 3D7vpkd was consistently observed with samples from children presenting with
329 severe malaria and uncomplicated malaria, and among acute convalescent samples.
330 This finding suggests that naturally-acquired antibodies to the IE surface are
331 predominantly PfEMP1-specific, which is supported by our previous data in PNG [36,37]
332 and Africa [35]. Low levels of antibodies to 3D7vpkd (which still expresses RIFIN,
333 STEVOR, and other antigens [35]) suggest that other IE surface antigens play a minor
334 role as antibody targets. Of note, antibodies specific to PfEMP1 were significantly higher

among children with uncomplicated malaria and associated with reduced odds of severe malaria. In contrast, there was no association between antibodies to 3D7vpkd and odds of severe malaria. Our data suggest that PfEMP1 is a major target of antibodies associated with protection from severe malaria, whereas antibodies to non-PfEMP1 antigens represent a less important component of protective immunity. Further investigation of other antigens is warranted in future studies.

Antibodies to recombinant 1E2 (IT4var19) PfEMP1 domains were also significantly higher in uncomplicated malaria, further supporting the contribution of PfEMP1 antibodies in immunity to severe malaria. Interestingly, only antibodies to two domains (DBLα2 and DBLγ6) were significantly associated with disease severity, suggesting these may be more important contributors of protective immunity. Collectively, our findings suggest that PfEMP1-specific antibodies protect against severe malaria in PNG children. Published work has suggested the importance of other CIDR domains in immunity against severe malaria [48,49], indicating that more detailed analyses are required to assess the relative contribution of specific DC8 PfEMP1 domains in protective immunity. A recent study in children in Mali (n=78 severe, n=73 uncomplicated cases) evaluated antibodies to PfEMP1 fragments using a microarray approach [50]. Antibodies were higher to recombinant PfEMP1 fragments in UM versus SM, but antibodies to the intact IE surface or antibody function were not evaluated. A potential limitation of the microarray approach is the use of protein fragments generated in an *E. coli* cell-free translation system; therefore, correct folding of PfEMP1 domains may be unlikely to occur, which may be important for antibody binding.

Antibodies to 3D7parental and 3D7vpkd were higher following convalescence; this was seen in uncomplicated and severe malaria, suggesting that naturally-acquired antibodies

to IE surface antigens are boosted upon infection. Interestingly, antibody levels to 1E2 (IT4var19) IEs were higher at convalescence for severe malaria only. This might reflect the expression of PfEMP1 types that are antigenically similar to the 1E2 (IT4var19) PfEMP1 in severe malaria, but this expression may be rare in uncomplicated malaria. Complementary research conducted with samples from this same clinical study profiled antibodies using a recombinant PfEMP1 domain array. They found that severe malaria resulted in the induction of antibodies to EPCR-binding CIDRα1 domains of PfEMP1 [Rambhatla et al, submitted to J Infect Dis].

Antibodies to IE surface antigens are believed to function, in part, by opsonizing IEs for clearance by phagocytes. Thus, we measured opsonic phagocytosis activity using undifferentiated THP-1 monocytes [35,44]. Opsonizing antibodies were significantly higher in uncomplicated malaria, suggesting a role for this mechanism in immunity. However, the modest extent of the difference suggests that other mechanisms are likely to play a role in immunity, such as inhibition of vascular adhesion, recruitment of complement, or interactions with other immune cells; these aspects need investigating in future studies. We showed that the level of opsonic phagocytosis activity was markedly reduced in 3D7vpkd compared to 3D7parental, further suggesting the importance of PfEMP1 as a major target of functional antibodies.

In conclusion, our study demonstrated a likely role of acquired antibodies to IE surface antigens in mediating protection against severe malaria in young children from PNG. We showed that PfEMP1 is a dominant target of naturally-acquired antibodies to the IE surface and a target of functional antibodies that promote opsonic phagocytosis of IEs. Furthermore, PfEMP1-specific antibodies were associated with protection against severe malaria in children, whereas antibodies to other IE surface antigens were not. These

findings significantly contribute to understanding malaria immunity and pathogenesis, and have implications for developing therapeutics or vaccines for preventing severe malaria.

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CONFLICT OF INTEREST

The authors declare no financial or commercial conflict of interest.

CORRESPONDING AUTHOR

James Beeson

Burnet Institute, 85 Commercial Road, Melbourne, VIC 3001, Australia

beeson@burnet.edu.au

412 Ph: 61-3-9282-2442

413 Fax: 61-3-9282-2100

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FIGURES/TABLES

Figure 1 Antibodies to the surface of *P. falciparum*-IEs are directed at PfEMP1

A, **C**. A representative selection of serum samples tested for antibodies to 3D7parental and 3D7vpkd parasites. Samples were collected from severe malaria (SM) and uncomplicated malaria (UM) at acute infection (**A**; SMA and UMA) and following convalescence (**C**; SMC, UMC). Samples from non-exposed Melbourne residents were used as a negative control (Control). IgG binding to 3D7vpkd was substantially reduced in all individuals. There was minimal background reactivity observed among sera from Melbourne residents. IgG binding levels are expressed as geometric mean fluorescence intensity (MFI) for all graphs; assay was performed once; bars represent MFI values of samples tested in singles.

B, **D**. Total IgG binding to the surface of erythrocytes infected with 3D7vpkd was substantially reduced compared to 3D7parental parasites in both severe and uncomplicated malaria groups at acute infection (**B**) and following convalescence (**D**). Assay was performed once; bars represent median and interquartile ranges of samples that were classified as antibody positive to 3D7parental (**B**, n=182/235 for SM and n=177/213 for UM; **D**, n=157/184 for SM and n=153/173 for UM); *p* values were calculated using a paired Wilcoxon signed rank test.

Figure 2 Antibodies to IE surface antigens are higher among young children presenting with uncomplicated malaria

A, **B**. The level of IgG binding to the surface of erythrocytes infected with 3D7parental and 3D7vpkd parasites was higher in samples from UM compared to SM at both acute (**A**) and convalescence (**B**). Assay was performed once; bars represent median and interquartile ranges of samples that were classified as antibody positive to 3D7parental

(**A**, n=182/235 for SM and n=177/213 for UM; **B**, n=157/184 for SM and n=153/173 for UM); *p* values were calculated using an unpaired Mann-Whitney test.

C, **D**. The level of IgG binding to the surface of erythrocytes infected with 1E2 (IT4var19) parasites was higher in samples from UM compared to SM at both acute (**C**) and convalescence (**D**). Assay was performed once; bars represent median and interquartile ranges (**C**, n=235 for SM and n=213 for UM; **D**, n=184 for SM and n=173 for UM); *p* value was calculated using an unpaired Mann-Whitney test.

Figure 3 Antibodies to IE surface antigens are higher among convalescent samples

A, **B**. Total IgG binding to the surface of erythrocytes infected with 3D7vpkd was substantially reduced compared to 3D7parental parasites at acute and convalescence for SM (**A**) and UM (**B**). Assay was performed once; bars represent median and interquartile ranges of samples that were classified as antibody positive to 3D7parental (**A**, n=182/235 for acute, n=157/184 for convalescence; **B**, n=177/213 for acute, n=153/173 for convalescence); *p* values were calculated using a paired Wilcoxon signed rank test.

C, **D**. The level of IgG binding to the surface of erythrocytes infected with 1E2 (IT4var19) parasites was higher in convalescence compared to acute samples for SM (**C**) and UM (**D**). Assay was performed once; bars represent median and interquartile ranges (**C**, n=235 for acute, n=184 for convalescence; **D**, n=213 for acute, n=173 for convalescence); *p* values were calculated using a paired Wilcoxon signed rank test.

Figure 4 Comparing the levels of antibodies to recombinant antigens of PfEMP1 in samples at acute infection

Total IgG binding to the recombinant antigens of PfEMP1 1E2 (IT4var19) is presented as (**A**) DBLα2, (**B**) CIDRα1, (**C**) DBLβ12 and (**D**) DBLγ6. Significantly higher antibody levels to UM compared to SM was only observed for (**A**) DBLα2 and (**D**) DBLγ6. Antibody levels are expressed as optical density (OD) values measured at 405nm. Assay was performed once; bars represent median and interquartile ranges of samples tested in duplicate (n=235 for SM and n=213 for UM); *p* values were calculated using an unpaired Mann-Whitney test.

Figure 5 Opsonic phagocytosis of IEs by undifferentiated THP-1 monocytes

A. A representative selection of serum samples collected during acute infection was tested for opsonic phagocytosis activity to 3D7parental and 3D7vpkd parasites. Assay was performed once (n=235 for SM, n=213 for UM); bars represent the mean level of phagocytosis as a percentage of positive control.

B. Opsonic phagocytosis activity of serum antibodies was markedly reduced with 3D7vpkd parasites compared to 3D7parental, for SM and UM. Bars represent the median and interquartile ranges of samples that were classified as antibody positive to 3D7parental (n=80/235 for SM and n=96/213 for UM); *p* values were calculated using a paired Wilcoxon signed rank test.

C. There was a higher level of opsonic phagocytosis activity with samples from UM compared to SM. Bars represent the median and interquartile ranges of samples that were classified as opsonic phagocytosis activity that is positive to 3D7parental (n=80/235 for SM and n=96/213 for UM); *p* value was calculated using an unpaired Mann Whitney test.

Table 1 Distribution of demographic characteristics of study participants at enrolment (acute infection)

Variables	Median (25^{th} – 75^{th} percentile) or Number (%)	
	Uncomplicated malaria (n=213)	Severe malaria (n=235)
Age (months)	42 (29–56)	40 (29–55)
Sex	127 (60%)	131 (56%)
Ethnicity		
Madang	174 (82%)	178 (76%)
Madang/Sepik	16 (8%)	23 (10%)
Other	13 (6%)	13 (6%)
Sepik	10 (5%)	20 (9%)

REFERENCES

1. World Health Organization. WORLD MALARIA REPORT 2015 Summary. **2016**; :1–32.

2. RTS,S Clinical Trials Partnership. Efficacy and safety of RTS,S/AS01 malaria vaccine with or without a booster dose in infants and children in Africa: final results of a phase 3, individually randomised, controlled trial. Lancet. **2015**; 386(9988):31–45.

3. Okiro EA, Al-Taiar A, Reyburn H, Idro R, Berkley JA, Snow RW. Age patterns of severe paediatric malaria and their relationship to *Plasmodium falciparum* transmission intensity. Malar J. **2009**; 8:4.

4. Chan J-A, Fowkes FJI, Beeson JG. Surface antigens of *Plasmodium falciparum*-infected erythrocytes as immune targets and malaria vaccine candidates. Cell Mol Life Sci. **2014**; 71(19):3633-57.

5. MacPherson GG, Warrell MJ, White NJ, Looareesuwan S, Warrell DA. Human cerebral malaria. A quantitative ultrastructural analysis of parasitized erythrocyte sequestration. Am J Pathol. **1985**; 119(3):385–401.

6. Aikawa M. Human cerebral malaria. Am J Trop Med Hyg. **1988**; 39(1):3–10.

7. Pongponratn E, Riganti M, Punpoowong B, Aikawa M. Microvascular sequestration of parasitized erythrocytes in human falciparum malaria: a pathological study. Am J Trop Med Hyg. **1991**; 44(2):168–175.

8. Abdi AI, Fegan G, Muthui M, et al. *Plasmodium falciparum* antigenic variation: relationships between widespread endothelial activation, parasite PfEMP1 expression and severe malaria. BMC Infect Dis; **2014**; 14(1):170.

9. Su XZ, Heatwole VM, Wertheimer SP, et al. The large diverse gene family *var* encodes proteins involved in cytoadherence and antigenic variation of *Plasmodium falciparum*-infected erythrocytes. Cell. **1995**; 82(1):89–100.

10. Lavstsen T, Salanti A, Jensen ATR, Arnot DE, Theander TG. Sub-grouping of *Plasmodium falciparum* 3D7 *var* genes based on sequence analysis of coding and non-coding regions. Malar J. **2003**; 2:27.

11. Tonkin-Hill GQ, Trianty L, Noviyanti R, et al. The *Plasmodium falciparum* transcriptome in severe malaria reveals altered expression of genes involved in important processes including surface antigen-encoding *var* genes. Plos Biol. **2018**; 16(3):e2004328.

12. Jensen ATR, Magistrado P, Sharp S, et al. *Plasmodium falciparum* associated with severe childhood malaria preferentially expresses PfEMP1 encoded by group A var genes. J Exp Med. **2004**; 199(9):1179–1190.

13. Rottmann M, Lavstsen T, Mugasa JP, et al. Differential expression of *var* gene groups is associated with morbidity caused by *Plasmodium falciparum* infection in Tanzanian children. Infection and Immunity. **2006**; 74(7):3904–3911.

14. Kaestli M, Cockburn IA, Cortés A, Baea K, Rowe JA, Beck H-P. Virulence of malaria is associated with differential expression of *Plasmodium falciparum var* gene subgroups in a case-control study. J Infect Dis. **2006**; 193(11):1567–1574.

15. Falk N, Kaestli M, Qi W, et al. Analysis of *Plasmodium falciparum var* genes expressed in children from Papua New Guinea. J Infect Dis. **2009**; 200(3):347–356.

16. Ghumra A, Semblat J-P, Ataíde R, et al. Induction of strain-transcending antibodies against Group A PfEMP1 surface antigens from virulent malaria parasites. PLoS Pathog. **2012**; 8(4):e1002665.

17. Turner GD, Morrison H, Jones M, et al. An immunohistochemical study of the pathology of fatal malaria. Evidence for widespread endothelial activation and a potential role for intercellular adhesion molecule-1 in cerebral sequestration. Am J Pathol. **1994**; 145(5):1057–1069.

18. Turner L, Lavstsen T, Berger SS, et al. Severe malaria is associated with parasite binding to endothelial protein C receptor. Nature. **2013**; 498(7455): 502-5.

19. Tuikue-Ndam N, Moussiliou A, Lavstsen T, et al. Parasites causing cerebral falciparum malaria bind multiple endothelial receptors and express EPCR and ICAM-1-binding PfEMP1. J Infect Dis. **2017**; 215(12):1918–1925.

20. Avril M, Tripathi AK, Brazier AJ, et al. A restricted subset of var genes mediates adherence of Plasmodium falciparum-infected erythrocytes to brain endothelial cells. Proceedings of the National Academy of Sciences. **2012**; 109(26):E1782–90.

21. Claessens A, Adams Y, Ghumra A, et al. A subset of group A-like var genes encodes the malaria parasite ligands for binding to human brain endothelial cells. Proceedings of the National Academy of Sciences. **2012**; 109(26):E1772-81.

22. Lavstsen T, Turner L, Saguti F, et al. *Plasmodium falciparum* erythrocyte membrane protein 1 domain cassettes 8 and 13 are associated with severe malaria in children. Proceedings of the National Academy of Sciences. **2012**; 109(26): E1791-800.

23. Lau CKY, Turner L, Jespersen JS, et al. Structural conservation despite huge sequence diversity allows EPCR binding by the PfEMP1 family implicated in severe childhood malaria. Cell Host Microbe. **2015**; 17(1):118–129.

24. Avril M, Brazier AJ, Melcher M, Sampath S, Smith JD. DC8 and DC13 *var* genes associated with severe malaria bind avidly to diverse endothelial cells. PLoS Pathog. **2013**; 9(6):e1003430.

25. Lennartz F, Adams Y, Bengtsson A, et al. Structure-guided identification of a family of dual receptor-binding PfEMP1 that is associated with cerebral malaria. Cell Host Microbe. **2017**; 21(3):403–414.

26. Avril M, Bernabeu M, Benjamin M, Brazier AJ, Smith JD. Interaction between Endothelial Protein C Receptor and Intercellular Adhesion Molecule 1 to mediate binding of *Plasmodium falciparum*-Infected erythrocytes to endothelial cells. MBio. **2016**; 7(4).

27. Nilsson Bark SK, Ahmad R, Dantzler K, et al. Quantitative proteomic profiling reveals novel *Plasmodium falciparum* surface antigens and possible vaccine candidates. Molecular & Cellular Proteomics. **2018**; 17(1):43–60.

28. Saito F, Hirayasu K, Satoh T, et al. Immune evasion of Plasmodium falciparum by RIFIN via inhibitory receptors. Nature. **2017**; 552(7683):101-105.

29. Kyes SA, Rowe JA, Kriek N, Newbold CI. Rifins: a second family of clonally variant proteins expressed on the surface of red cells infected with *Plasmodium falciparum*. Proc Natl Acad Sci USA. **1999**; 96(16):9333–9338.

30. Kaviratne M, Khan SM, Jarra W, Preiser PR. Small variant STEVOR antigen is uniquely located within Maurer's clefts in *Plasmodium falciparum*-infected red blood cells. Eukaryotic Cell. **2002**; 1(6):926–935.

31. Winter G, Kawai S, Haeggström M, et al. SURFIN is a polymorphic antigen expressed on *Plasmodium falciparum* merozoites and infected erythrocytes. J Exp Med. **2005**; 201(11):1853–1863.

32. Nielsen MA, Staalsoe T, Kurtzhals JAL, et al. *Plasmodium falciparum* variant surface antigen expression varies between isolates causing severe and nonsevere malaria and is modified by acquired immunity. J Immunol. **2002**; 168(7):3444–3450.

33. Griffin JT, Hollingsworth TD, Reyburn H, Drakeley CJ, Riley EM, Ghani AC. Gradual acquisition of immunity to severe malaria with increasing exposure. Proc Biol Sci. **2015**; 282(1801):20142657.

34. Gupta S, Snow RW, Donnelly CA, Marsh K, Newbold C. Immunity to non-cerebral severe malaria is acquired after one or two infections. Nat Med. **1999**; 5(3):340–343.

35. Chan J-A, Howell KB, Reiling L, et al. Targets of antibodies against *Plasmodium falciparum*-infected erythrocytes in malaria immunity. J Clin Invest. **2012**; 122(9):3227-38.

36. Chan J-A, Howell KB, Langer C, et al. A single point in protein trafficking by *Plasmodium falciparum* determines the expression of major antigens on the surface of infected erythrocytes targeted by human antibodies. Cell Mol Life Sci. **2016**; 73(21):4141-58.

37. Chan J-A, Stanisic DI, Duffy MF, et al. Patterns of protective associations differ for antibodies to *P. falciparum*-infected erythrocytes and merozoites in immunity against malaria in children. Eur J Immunol. **2017**;47(12):2124-2136.

38. Turner L, Lavstsen T, Mmbando BP, et al. IgG antibodies to endothelial protein C receptor-binding cysteine-rich interdomain region domains of *Plasmodium falciparum* erythrocyte membrane protein 1 are acquired early in life in individuals exposed to malaria. Infection and Immunity. **2015**; 83(8):3096–3103.

39. Cham GKK, Turner L, Lusingu J, et al. Sequential, ordered acquisition of antibodies to *Plasmodium falciparum* erythrocyte membrane protein 1 domains. J Immunol. **2009**; 183(5):3356–3363.

40. Duffy MF, Noviyanti R, Tsuboi T, et al. Differences in PfEMP1s recognized by antibodies from patients with uncomplicated or severe malaria. Malar J. **2016**; 15(1):258.

41. Manning L, Laman M, Law I, et al. Features and prognosis of severe malaria caused by *Plasmodium falciparum*, *Plasmodium vivax* and mixed *Plasmodium* species in Papua New Guinean children. PLoS ONE. **2011**; 6(12):e29203.

42. Voss TS, Healer J, Marty AJ, et al. A *var* gene promoter controls allelic exclusion of virulence genes in Plasmodium falciparum malaria. Nature. **2006**; 439(7079):1004–1008.

627 43. Reiling L, Richards JS, Barry AE, et al. Evidence that the erythrocyte invasion
628 ligand PfRh2 is a Target of Protective Immunity against Plasmodium falciparum
629 Malaria. J Immunol. **2010**; 185(10):6157–6167.

630 44. Ataíde R, Hasang W, Wilson DW, et al. Using an improved phagocytosis assay to
631 evaluate the effect of HIV on specific antibodies to pregnancy-associated malaria.
632 PLoS ONE. **2010**; 5(5):e10807.

633 45. Jespersen JS, Wang CW, Mkumbaye SI, et al. *Plasmodium falciparum var* genes
634 expressed in children with severe malaria encode CIDRα1 domains. EMBO Mol
635 Med. **2016**; 8(8):839–850.

636 46. Mkumbaye SI, Wang CW, Lyimo E, et al. The severity of *Plasmodium falciparum*
637 infection is associated with transcript kevels of *var* genes encoding Endothelial
638 Protein C Receptor-Binding *P. falciparum* Erythrocyte Membrane Protein 1.
639 Infection and Immunity. **2017**; 85(4).

640 47. Shabani E, Hanisch B, Opoka RO, et al. Plasmodium falciparum EPCR-binding
641 PfEMP1 expression increases with malaria disease severity and is elevated in
642 retinopathy negative cerebral malaria. BMC Med. **2017**; 15(1):183.

643 48. Nunes-Silva S, Dechavanne S, Moussiliou A, et al. Beninese children with
644 cerebral malaria do not develop humoral immunity against the IT4-VAR19-DC8
645 PfEMP1 variant linked to EPCR and brain endothelial binding. Malar J. **2015**;
646 14(1):493.

647 49. Abdi AI, Hodgson SH, Muthui MK, et al. *Plasmodium falciparum* malaria parasite
648 var gene expression is modified by host antibodies: longitudinal evidence from

controlled infections of Kenyan adults with varying natural exposure. BMC Infect Dis. **2017**; 17(1):585.

50. Travassos MA, Niangaly A, Bailey JA, et al. Children with cerebral malaria or severe malarial anaemia lack immunity to distinct variant surface antigen subsets. Sci Rep. Springer US; **2018**; :1–14.