1   **Title: Identifying targets of protective antibodies against severe malaria in Papua,**

2   **Indonesia using locally expressed domains of *Plasmodium falciparum* Erythrocyte**

3   **Membrane Protein 1.**

4   **Short title: PfEMP1 antibodies and malaria severity in Papua**

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46 **Abstract** *(221/250 words)*

47 *Plasmodium falciparum* erythrocyte membrane protein 1 (PfEMP1), a diverse family of multi-

48 domain proteins expressed on the surface of malaria-infected erythrocytes, is an important

49 target of protective immunity against malaria. Our group recently studied transcription of the

50 *var* genes encoding PfEMP1 in individuals from Papua, Indonesia with severe or

51 uncomplicated malaria.

52 We cloned and expressed domains from 32 PfEMP1s including 22 that were upregulated in

53 severe malaria and 10 that were upregulated in uncomplicated malaria, using a wheat germ

54 cell-free expression system. We used Luminex technology to measure IgG antibodies to these

55 32 domains and control proteins in 63 individuals (11 children). At presentation to hospital,

56 levels of antibodies to PfEMP1 domains were either higher in uncomplicated malaria or were

57 not significantly different between groups. Using principal components analysis, antibodies

58 to three of 32 domains were highly discriminatory between groups. These included two

59 domains upregulated in severe malaria, a DBLβ13 domain and a CIDRα1.6 domain (which has

60 been previously implicated in severe malaria pathogenesis), and a DBLδ domain that was

61 upregulated in uncomplicated malaria. Antibody to control non-PfEMP1 antigens did not

62 differ with disease severity.

63 Antibodies to PfEMP1 domains differ with malaria severity. Lack of antibodies to locally

64 expressed PfEMP1 types, including both domains previously associated with severe malaria

65 and newly identified targets, may in part explain malaria severity in Papuan adults.

66 **Importance** *(150/150 words)*

67 Severe *Plasmodium falciparum* malaria kills many African children, and lack of antibody

68 immunity predisposes to severe disease. A critical antibody target is the *P. falciparum*

69 erythrocyte membrane 1 (PfEMP1) family of multidomain proteins, which are expressed on

70 the infected erythrocyte surface and mediate parasite sequestration in deep organs.

71 We previously identified *var* genes encoding PfEMP1 that were differentially expressed

72 between severe and uncomplicated malaria in Papua, Indonesia. Here, we have expressed

73 domains from 32 of these PfEMP1s and measured IgG antibody responses to them in Papuan

74 adults and children. Using Principal Component Analysis, IgG antibodies to three domains

75 distinguished between severe and uncomplicated malaria and were higher in uncomplicated

76 malaria. Domains included CIDRα1.6, implicated in severe malaria; a DBLβ13 domain; and a

77 DBLδ domain of unknown function.

78 Immunity to locally relevant PfEMP1 domains may protect from severe malaria. Targets of

79 immunity show important overlap between Asian adults and African children.

80

81

Introduction

Malaria kills over 400,000 people each year, primarily young African children infected with *Plasmodium falciparum* (1). Conversely in the Asia-Pacific region, lower exposure to infection in early life results in a greater proportion of severe malaria occurring in young adults. The clinical manifestations of severe malaria differ between children and adults (2), although the pathology of fatal malaria appears broadly similar (3, 4). Recent studies have begun to compare the characteristics of parasites causing severe malaria and how these differ between children and adults (5).

Among the key pathogenic characteristics of *P. falciparum* is the transcription of *var* genes which encode the *P. falciparum* erythrocyte membrane protein 1 (PfEMP1) family of proteins. There are ~60 *var* genes per parasite genome (6), with only limited overlap between genomes, but they can be classified into groups A, B and C based on upstream promoter sequences and chromosomal location (7). Group A and chimeric group B/A *var* genes have been most consistently associated with severe malaria (8-10), and expression of conserved sets of PfEMP1 variants based on *var* gene groupings has been associated with high pathogenicity and low host immunity (11).

Expressed on the surface of mature infected erythrocytes (IEs), PfEMP1s are large, 200-450 kDa proteins which mediate sequestration of IEs in the deep vasculature through adhesion to endothelial receptors. Structurally, the extracellular portion of PfEMP1 includes varying numbers of Duffy binding-like (DBL) domains and cysteine-rich interdomain regions (CIDRs). DBL domains form six major classes, DBL α, β, γ, δ, ε and ζ and there are five sub-classes of CIDR domains α, β, γ, δ and pam. Of these, some domains have known adhesive functions. For example, CIDRα1 domains mediate adhesion to endothelial protein C receptor (EPCR)

105 (12), CIDRα2-6 domains often bind to CD36 (13, 14), and DBLβ domains often bind to

106 intercellular adhesion molecule 1 (ICAM-1) (15, 16). Within PfEMP1 sequences, domain

107 cassettes (DC) are conserved arrangements of two or more PfEMP1 domains which can be

108 predicted from one another and may indicate receptor specificity (17).

109 PfEMP1s are major targets of protective immunity against severe and uncomplicated malaria

110 (18-21). Antibody immunity against PfEMP1 domains belonging to different *var* groups is

111 acquired with age in an ordered fashion: first against the group A variants, followed by group

112 B and C PfEMP1 types (22, 23). Immunity to severe disease is acquired relatively rapidly,

113 consistent with the existence of a restricted set of severe malaria-associated virulent antigens

114 (24), most notably PfEMP1 (21). Recent studies have attempted to identify PfEMP1 domains

115 that are major targets of antibodies that protect from severe malaria, together with PfEMP1

116 antibody responses that are boosted following severe malaria in children (25-30). Limited

117 studies have investigated the role of antibodies in protecting adults from severe malaria,

118 including severe malaria in pregnancy (31, 32).

119 To date most studies of PfEMP1 antibodies have used domains expressed from the genomes

120 of laboratory-adapted isolates, or from genomic DNA of parasites in a population (22, 23, 27-

121 30, 33); one recent study identified PfEMP1 peptides from proteomic analyses of clinical

122 isolates (34). To ensure that PfEMP1 domain targets are representative of those expressed by

123 parasites circulating in the community, we used domains identified as differentially expressed

124 between severe and uncomplicated malaria by next generation sequencing of clinical isolates

125 from Papua, Indonesia (35). Thirty-two PfEMP1 domains were cloned and expressed, and

126 antibody responses to these domains were measured in the cognate population of Papuan

127 individuals with severe or uncomplicated malaria at presentation. In univariate analyses

128 antibodies to many of these proteins were higher in uncomplicated than in severe malaria.

129 Using principal components analysis (PCA), antibody to three of these domains partly

130 separated the participants by disease severity.

131 **Results**

132 **Study population**

133 Plasma samples were collected on presentation from 28 patients with severe malaria and 35

134 with uncomplicated malaria (Table 1). Participants with severe malaria had a median age of

135 29 years, 6 (21.4%) were children <16 years old and 11 (39.3%) were female. Individuals with

136 uncomplicated malaria had a median age of 22.5 years, 5 (14.3%) were children and 16

137 (45.7%) were females. In total 23 of 28 (82.2%) patients with severe malaria presented with

138 a single manifestation of severe malaria (Table 2).

139 **PfEMP1 domains cloned, sequenced and expressed**

140 Thirty-two PfEMP1 domains were successfully expressed using the wheat germ cell-free

141 system (WGCFS) including 22 of 28 selected domains upregulated in severe malaria and 10 of

142 16 selected domains upregulated in uncomplicated malaria, together with AMA-1 and CSP.

143 This yielded ~ 11-200 μg of purified proteins with an N-terminal 6-Histidine tag. The

144 differentially expressed domains are illustrated in Table 3, together with their domain types

145 and the DCs to which they have been allocated. Primers used to clone domains, outcome of

146 cloning and expression and resulting DNA sequences can be found in Table S1. Where

147 possible, expressed domains were assigned to a DC (Table 3); some domains lie outside of

148 described DCs (35).

149 Sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis gels for the proteins

150 expressed are illustrated in Figure S1. The multiple bands observed for some proteins may

151 represent truncated forms of the expressed proteins produced during affinity purification due

152 to natural translational arrest during translation (36).

153 **Antibody levels against individual PfEMP1 domains**

154 In univariable analyses, when Melbourne controls and Papuans with severe or uncomplicated

155 malaria were analysed together, antibody levels differed significantly between groups (Table

156 S2), being lowest in Melbourne controls. Antibody levels were significantly higher in

157 individuals with uncomplicated malaria compared to severe malaria against 15 of 22 severe

158 malaria associated PfEMP1 domains. Similar trends, although not statistically significant, were

159 observed for six of the other proteins (Figure 1). Antibody levels towards SM18 CIDRα1.1 were

160 higher in severe malaria than in uncomplicated malaria. Antibody levels were also significantly

161 higher in uncomplicated malaria for seven of ten domains that were upregulated in

162 individuals with uncomplicated malaria, and showed similar trends for the three remaining

163 proteins (Figure 2).

164 *P. falciparum* control proteins, AMA-1 and CSP were included twice in the analysis. In one

165 assay, antibodies to CSP were slightly higher in uncomplicated malaria ($p = 0.044$; Figure 2).

166 Antibodies against tetanus toxin did not differ between malaria groups (data not shown), and

167 malaria-naïve Melbourne controls had low levels of antibody to all malaria antigens.

168 **Multivariate analysis using LASSO penalized regression.**

169 To further identify the key antibody targets that differentiate severe from uncomplicated

170 malaria, responses to all 32 PfEMP1 domains (and 2 control proteins) were included in LASSO

171 penalized regression analysis. Three proteins were selected, and PCA was applied to visualize

172 separation of the population in two dimensions (Figure 3 A). Antibody levels in individuals

173 with severe malaria (blue dots) cluster somewhat separately from those with uncomplicated

174   malaria (red dots) (leave-one-out cross validation, LOOCV = 71.4%, combined X variance =

175   52.8%; Figure 3 A). The three domains that best predicted separation of the groups by disease

176   severity included two domains upregulated in severe malaria (SM19-CIDRα1.6 and SM25-

177   DBLβ13) and one domain upregulated in uncomplicated malaria (UM2-DBLδ1) (Figure 3 B).

178   1000-fold resampling of our data set yielded the same three proteins in all instances, and in

179   about 12% of cases also identified one additional protein as contributing significantly (Figure

180   S2). For each domain, participants with uncomplicated malaria had significantly higher

181   antibody levels as compared to those with severe malaria in univariate analyses (Figure 1,

182   Figure 2). Antibodies to AMA-1 and CSP did not contribute to the separation of groups. When

183   Melbourne controls were included in the LASSO analysis, antibody to seven domains best

184   separated the three groups, including the three domains identified above (Figure S3A, B). In

185   the associated PCA, controls were separated from malaria infected subjects across the first

186   principal component with a variance of 78% (Figure S3C).

187   **Relationship between antibody levels and var transcription**

188   We examined whether there was a relationship between the levels of antibodies to a protein

189   and the level of transcripts encoding the protein amongst study participants. There was no

190   significant correlation for all patients aggregated, or for patients segregated into severe and

191   uncomplicated groups. Additionally, levels of antibodies did not differ between proteins from

192   genes that were transcribed and those that were not transcribed within either the severe or

193   uncomplicated patient groups. Moreover, transcription of the genes encoding the two

194   proteins that were upregulated in severe malaria patients and that best separated antibody

195   responses of severe from uncomplicated malaria did not differ significantly from the

196   transcription of the genes encoding the other 20 proteins that were also upregulated in

197   severe malaria and analysed for antibody response (data not shown). UM2-DBLδ1 was the

198   only protein that was transcribed at higher levels in uncomplicated malaria than in severe

199   malaria and that had levels of antibody which contributed to separating the groups.

200   Examining the transcription data, only one patient with severe malaria expressed UM2-

201   DBLδ1, whereas nine uncomplicated malaria patients did, sometimes at high levels (data not

202   shown).

203

204   **Discussion**

205   Evidence from multiple sources indicates that antibody immunity to PfEMP1 may be

206   protective against severe malaria (25-30). We previously identified dominant PfEMP1 types

207   expressed in severe malaria in Papua, using next generation sequencing and novel

208   bioinformatic analyses, which offers significant advantages over quantitative polymerase

209   chain reaction (qPCR) for assessment of *var* expression in clinical isolates. Absolute sequence

210   abundance is measured, and biases in amplification related to varying degrees of binding to

211   the qPCR primers used are avoided. Using next generation sequencing outputs, we identified

212   PfEMP1 domains that were differentially transcribed in severe or uncomplicated malaria in

213   individuals from Papua, Indonesia (35). We expressed 32 of these PfEMP1 domains, including

214   22 that were upregulated in severe malaria and 10 upregulated in uncomplicated malaria,

215   and examined whether IgG antibody to these domains could distinguish between individuals

216   from the same setting with uncomplicated and severe malaria.

217   In univariable analyses, antibodies to 15 of 22 severe malaria-associated domains and seven

218   of 10 uncomplicated malaria-associated domains were significantly higher in uncomplicated

219   than severe malaria, while responses to nine of the other ten domains did not differ

220   significantly between groups. Antibodies against control antigens including AMA-1 and CSP

221 did not differ significantly between groups. These findings suggest that antibody to locally

222 prevalent PfEMP1 types, but not to control antigens, may be important in protection from

223 severe malaria.

224 To determine which PfEMP1 domains might be most important in protection, levels of

225 antibody to PfEMP1 domains and control antigens were analysed using PCA and LASSO

226 regularized logistic regression. Using LASSO, three antibody features best discriminated

227 between groups, and IgG antibody to these three domains was able to partially separate the

228 patient groups. These included a CIDRα1.6 domain, a DBLβ13 domain and a DBLδ1 domain,

229 and antibody to all three domains was higher in uncomplicated malaria subjects.

230 CIDRα1 domains are important targets of immunity against severe malaria. Transcription of

231 CIDRα1 domains is upregulated in severe malaria (10, 16, 37-39), and children with severe

232 malaria may lack antibody to CIDRα1 proteins and develop antibodies to them in

233 convalescence (27, 28). The clinical importance of the DBLβ13 domains is less clear. Although

234 many DBLβ domains bind ICAM-1, the sole DBLβ13 domain thus far tested did not (40), and

235 its function is unknown. Furthermore the DBLβ13 domain expressed for the current study

236 lacked the sequence motifs required for ICAM-1 binding structural features and direct

237 interaction with ICAM-1 (16) and thus was unlikely to mediate adhesion to ICAM-1. Similarly,

238 the function of DBLδ1 is unknown. Whilst efforts to date have focused on domains of known

239 function and those located in the N-terminal region of PfEMP1 as targets of immunity,

240 antibody to other domains may be equally important in protection, perhaps by interfering

241 with correct PfEMP1 folding or display, or by targeting IE for phagocytic clearance.

242 DBLδ domains form the most diverse domain family (41), and their functional role requires

243 further study. By integrating RNA-Seq and serology data we were able to identify that the

244   DBLδ1 was rarely transcribed in severe malaria, suggesting that differences in antibodies

245   between groups were most probably due to lack of exposure in severe malaria. It is possible

246   that variants expressing this domain only emerge after development of immunity to more

247   virulent types. A lower virulence and potential for persistence have similarly been postulated

248   for group C *var* genes (11, 32, 42).

249   One driver of severe malaria risk in children is the emergence of IEs expressing PfEMP1

250   variants that can sequester efficiently in the deep vasculature, and to which the host lacks

251   immunity (reviewed in (43)). While studies have related immunity to PfEMP1 domains to

252   susceptibility to severe disease in children (25-28, 30), the role of antibody in protecting

253   adults from severe malaria is less well studied. Over 80% of our study participants were

254   adults, likely because malaria transmission in Timika is unstable (44) and internal migration is

255   common, so many adults lack established malaria immunity. Our observations, and a previous

256   study (32), indicate that Papuan adults with severe malaria have overall lower recognition of

257   PfEMP1 proteins than adults with uncomplicated malaria, and the domains that best

258   distinguish groups have both similarities to those reported as important in severe malaria in

259   African children, and some differences. This could be due to our novel approach of identifying

260   transcribed locally relevant domains for study.

261   Relatively little is known about PfEMP1 expression outside of Africa but a recent study in

262   young children from PNG showed that lack of antibody to DBLα domains isolated from gDNA

263   of isolates from the same origin predicted susceptibility to severe malaria (30). A study of *var*

264   repertoires from Africa and Asia shows evidence of significant co-clustering of sequences

265   from both continents, indicating substantial intercontinental gene flow (41). However, a

266   global study of DBLα tags showed that although Papua New Guinean and Asian sequences

267   were more closely related to African than South American isolates, the Papuan New Guinean

268   sequences were much more closely related to each other than to DBLα sequences from

269   elsewhere in the world (45). Thus, whether PfEMP1 domains circulating in a specific area are

270   more important targets of protective immunity than globally conserved domains warrants

271   further study.

272   **Strengths and Limitations**

273   A key feature of our study is that we expressed PfEMP1 domains that were highly transcribed

274   in malaria patients and tested the same population for immunity to the cognate domain (35).

275   We did not demonstrate an association between expression levels of the PfEMP1s and the

276   levels of antibodies to the PfEMP1s in the same patients. It may be necessary to investigate

277   antibodies that bind to smaller PfEMP1 peptides upregulated in severe malaria (35) to

278   demonstrate that antibodies to virulence-associated PfEMP1 sequences specifically protect

279   patients from parasites expressing the same PfEMP1s. However, as we show for DBLδ, these

280   complementary data have the potential to discriminate between antibody responses

281   associated with protection from parasites expressing PfEMP1s that are upregulated in severe

282   disease and antibody responses due to parasites selectively expressing other PfEMP1s in

283   uncomplicated malaria. Both of these phenomena manifest as increased levels of PfEMP1-

284   specific antibody in uncomplicated compared to severe disease. Integrating transcriptomics

285   and serology can reveal not only potential targets of protective immunity, but also deepen

286   our understanding of regulation of PfEMP1 expression in populations with differing levels of

287   immunity.

288   Two issues should be borne in mind in interpreting the results. First, the duration of illness

289   was not recorded for most participants, and the relationship between duration of illness and

290   duration of infection is highly variable. It is possible that differences in antibody levels

291   between the groups could be driven in part by differences in duration of the current infection,

292   if participants with UM had longer illness or infection duration than those with SM. Second,

293   the progression of clinical malaria from uncomplicated to severe is highly unpredictable, and

294   subjects with uncomplicated malaria might have progressed to severe disease, if the illness

295   episode had not been treated. Other host factors beyond antibody response may also affect

296   the risk or timing of disease progression. It appears unlikely that these factors would explain

297   the higher levels of antibody to a wide range of PfEMP1 proteins in uncomplicated malaria as

298   seen here, but future studies should attempt to control for duration of infection or symptoms

299   and for other relevant host factors.

300   The study had relatively small numbers precluding analysis of antibody responses in relation

301   to individual severe malaria syndromes, such as the observation that cerebral malaria is

302   associated with PfEMP1 types that bind to both ICAM-1 and EPCR (16). We did not have

303   convalescent samples, to examine antibodies to PfEMP1 targets that develop following

304   severe or uncomplicated malaria. Because our approach to identify domains for expression

305   was based on transcription rather than imputed function, we had relatively few examples of

306   some domain types, with (for example) three CIDRα1 domains. To date we have only

307   measured IgG antibody to the expressed domains, and more detailed analysis of antibody

308   isotype or functional activity might yield different results (46). Direct comparison of the

309   targets of protective antibody in adults and children from different geographical regions

310   should be considered, together with expanding the assays to measure functional properties

311   of antibody such as IgG subclass responses and engagement with complement and Fc

312   receptors on immune cells (47).

313

Conclusion

In Papua, Indonesia, antibody to locally relevant PfEMP1 domains was often significantly higher in individuals with uncomplicated than severe malaria, suggesting PfEMP1 antibody may protect against severe malaria. Using PCA and LASSO, we were able to partly separate groups based on antibody, and three proteins contributed to this separation, including a CIDRα1.6 domain, associated with protective immunity in African children, but also two domain types not previously associated with either biological function or immune response. This work significantly expands our knowledge of antibody immunity to PfEMP1 in the Asia-Pacific region and in adults and indicates the value of unbiased approaches to identifying differentially transcribed PfEMP1 domains, and of assessing their roles in protective immunity. To develop a PfEMP1 based vaccine (48), it will be necessary to identify key targets, features and functions of protective antibodies, and to understand the acquisition, boosting and maintenance of such antibody responses in malaria-exposed populations (49).

Methods

Clinical subjects

Study participants presented with severe or uncomplicated malaria at a healthcare facility in Timika, Papua Province, Indonesia (32, 35). In Timika, malaria transmission is unstable, and the estimated annual parasite incidence is 450 per 1000 population. Symptomatic malaria occurs in all age groups (44). Following informed consent, a venous blood sample was collected from 28 patients with severe malaria and 35 patients with uncomplicated malaria on presentation for antibody studies and *var* gene analysis (32, 35). Plasma was separated and stored at -20°C. Severe malaria was defined as peripheral parasitaemia with at least one

337 modified World Health Organization (WHO) criterion of severity (50). In 26 of the 28 patients

338 with severe malaria, parasite densities were above 1000/µL, a threshold that predicts clinical

339 disease in northern Papua [49]. Thus, incidental parasitemia is unlikely in these 26 severe

340 malaria patients but cannot be excluded in two severe malaria patients with parasitemia

341 below 1000/µl.

342 Of 63 subjects, 44 were used for *var* analysis and all 63 were available for antibody studies.

343 Reasons why RNA was not analysed included no sample (3 cases), sample used for assay

344 optimisation (5 cases) and insufficient RNA extracted (11 cases). *Var* analysis was successfully

345 performed on 23 of 27 severe malaria samples and 21 of 28 uncomplicated malaria samples

346 (35). Plasma samples collected at presentation were used to measure antibody levels against

347 PfEMP1 domains, *P. falciparum* control proteins and tetanus toxin as an additional control.

348 Serum samples from nine unexposed Melbourne blood donors were used as negative controls

349 for antibody studies.

350 **Identifying PfEMP1 domains of interest**

351 Previously we analysed RNA from IEs from 23 patients with severe malaria and 21 patients

352 with uncomplicated malaria by Illumina RNAseq and *de novo* assembled *var* transcripts (35).

353 4449 individual *var* transcripts were assembled and annotated with the domain models (17)

354 available at ENA and NCBI as bioproject PRJEB20632. A differential expression analysis of

355 these domain classes was performed and identified 16 domains that were upregulated and

356 four that were downregulated in severe malaria. These domain classes were defined by

357 bootstrapped phylogenetic trees (17) and thus include many sequences at low pair-wise

358 sequence identity. To identify more conserved domain sequences that were upregulated in

359 severe malaria the annotated domain sequences were re-clustered hierarchically at different

360   sequence identity levels. This identified 15 clusters of domain subtypes that were upregulated

361   and 55 domains that were downregulated in severe malaria. Twenty-eight unique domains

362   were identified out of the 31 domains upregulated in severe malaria across these two

363   analyses.

364   **Selection of patient isolates expressing PfEMP1 domains of interest and primer design**

365   We attempted to amplify and clone representatives of every domain that was upregulated in

366   severe malaria (adjusted p-value <0.05 and log fold change ≥ 2) from the cognate patient

367   genomic DNA (gDNA) (35). Twenty two of the 28 different PfEMP1 domains upregulated in

368   severe malaria were successfully expressed, the domains that could not be successfully

369   amplified or cloned included DBLα1.2, DBLα2, DBLγ, DBLδ1, DBLε2 and CIDRγ9 domains (35).

370   A tandem CIDRα2.6-DBLβ5 sequence was also cloned because it was upregulated in

371   additional whole transcript differential expression analyses (35).

372   To select domains with upregulated expression in uncomplicated malaria for cloning, we

373   attempted to amplify the 15 domains most upregulated in uncomplicated malaria from the

374   hierarchical analysis (adjusted p value < 0.05). Ten of these domains could not be successfully

375   amplified or cloned from malaria patient DNA. The lower success in amplification of these

376   sequences probably reflected the lower parasitemia and thus parasite DNA in uncomplicated

377   compared to severe malaria. The CIDRα1 sequence that was most downregulated in severe

378   malaria was also selected. This CIDRα1.7 was considered an important control for the cloned

379   CIDRα1.1 and CIDRα1.6 sequences that were upregulated in severe malaria.

380   Primers of length 15-33 nucleotides with melting temperatures of 51-60°C were designed

381   using Geneious R10 (Biomatters, New Zealand) (Supplementary Table 1). The primers

382   included flanking restriction sites for XhoI (or BamHI) in the forward primer and XmaI (or NotI)

383   in the reverse primer. A stop codon in the reverse primer before the restriction site

384   terminated the protein translation. Wherever possible protein sequences included an even

385   number of cysteine residues to facilitate protein folding.

386   Sequences for apical membrane antigen 1 (AMA-1) and circumsporozoite protein (CSP) were

387   obtained from PlasmoDB and gDNA from one of the patients with severe malaria was used

388   for cloning and expression. These proteins served as controls for the wheat germ cell-free

389   protein synthesis systems (WGCFS) method of protein production and as control markers of

390   malaria exposure in the population.

391   **Amplification and purification of domains of interest**

392   DNA sequences for the domains of interest were amplified from gDNA of parasites from

393   malaria infected individuals by PCR using primers listed (Supplementary Table I) and KAPAHiFi

394   polymerase (Roche, Basel, Switzerland) using the Eppendorf Gradient system and Applied

395   Biosystems GeneAmp® PCR system 9700. Thirty cycles of amplification were performed, with

396   denaturation at 94°C for 60 seconds, annealing at 50-59°C (depending on primer) for 60

397   seconds and extension at 68°C for 110-120 seconds.

398   **Purification of restriction enzyme (RE) digested DNA of interest and bacterial**

399   **transformation**

400   PCR products were separated by agarose gel electrophoresis and purified from the gel. The

401   purified DNA and the plasmid pEU-E01-His-TEV-MCS (CellFree Sciences, Matsuyama, Japan)

402   (51) were digested using the restriction enzymes XhoI and XmaI or BamHI and NotI (New

403   England BioLabs, Ipswich MA, USA), further purified and then ligated using T4 DNA ligase

404   (New England BioLabs).

405   Five µl of ligated products were transformed into 50 µl of ultra-competent *E. coli* XL-10-Gold

406   (Agilent Technologies, Santa Clara CA, USA) cells by incubation on ice for 30 minutes then

407   heat shock at 42°C for 40 seconds followed by incubation on ice for a further 5 minutes. Plated

408   clones were screened for the recombinant plasmids by PCR using vector-specific primers. The

409   recombinant plasmid sequences in recovered clones were confirmed by Sanger sequencing.

410   **Wheat germ cell-free protein expression**

411   Recombinant pEU wheat germ expression vector plasmids encoding the domains of interest

412   were expressed using the WGCFS at Ehime University using WGCFS reagents purchased

413   from CellFree Sciences, as previously described (52, 53). The WGCFS uses the pEU

414   expression vector which contains a SP6 promoter to drive RNA synthesis and an E01

415   translational enhancer to induce cap-independent translation. The in vitro transcribed RNA

416   is used as a template for protein synthesis in a "Buffered Substrate Solution" which includes

417   the amino acids, a DTT-based redox system, and a creatine kinase driving energy supply.

418   Transcription and translation reactions were performed on a 6 ml scale in a Protemist DTII

419   robotic protein synthesizer followed by purification of His-tagged proteins.

420

421   **Multiplex assays**

422   Protein coupling was carried out as previously described (54, 55) with a few modifications.

423   Bio-Plex magnetic carboxylated microspheres (Bio-Rad, Hercules CA, USA, 1.25×10^7

424   beads/ml) were resuspended by vortexing and sonication for 30 seconds and washed using

425   the Bio-Rad magnetic separator. 100 µl of each bead suspension containing 1.25×10^6 beads

426   were activated using 80 µl activation buffer (0.1 M NaH_2PO_4, pH 6.2) and incubated with 10

427   µl each of 50 mg/ml N-hydroxysulfosuccinimide (Thermo Fisher Scientific, Waltham MA, USA)

428 and 50 mg/ml of Pierce™ Premium Grade EDC (1-ethyl-3-(3-dimethylaminopropyl)

429 carbodiimide hydrochloride; Thermo Fisher Scientific) in the dark for 30 minutes at RT with

430 rotation. Beads were washed twice with 150 µl coupling buffer (0.1 M 2-(N-morpholino)

431 ethanesulfonic acid, pH 6.1) (Sigma-Aldrich, St Louis MO, USA). After adding 5-12 µg of protein

432 to the activated beads they were vortexed and incubated for 3 hours in the dark with rotation.

433 The beads were washed with phosphate buffered saline (PBS) and incubated with blocking

434 buffer (0.1% Bovine Serum Albumin, 0.02% Tween 20, 0.05% sodium azide in PBS) for 30

435 minutes in the dark at ambient temperature with rotation, then washed and resuspended in

436 sterile storage buffer (0.05% sodium azide in PBS). Protein-coupled beads were stored at 4°C

437 for up to one month or at -80°C for up to 6 months.

438 Levels of IgG antibodies to the antigen-coupled microspheres were measured as previously

439 described (54, 55). Briefly, bead cocktails were diluted in assay buffer (1% BSA in PBS) to a

440 concentration of 10 beads/µl for each bead. In 96-well flat-bottom black plates (Bio-Plex

441 Pro™, Bio-Rad), 50 µl of bead suspension was mixed with 50 µl of test plasma (diluted 1:50 in

442 PBS) in each well. Negative controls included serum from Melbourne naïve individuals and a

443 no-plasma well. Pooled malaria-reactive plasma formed a positive control, while a 5-fold,

444 nine-point serial dilution of total IgG (Sigma-Aldrich), starting with 500 µg/ml concentration

445 was used for the standards.

446 Plates were incubated overnight at 4°C on a plate shaker in the dark, spun and washed with

447 0.1% Tween in PBS (PBS-Tween) using the BioPlex Pro II wash station. 50 µl of 1.3 µg/ml

448 mouse anti-human IgG-PE (Southern Biotech, Birmingham AL, USA) diluted in assay buffer

449 was added. After 2 hours incubation in the dark at RT on the plate shaker, the plates were

450 washed twice with PBS-Tween, incubated in sheath fluid for 10 minutes in the dark, on a plate

451   shaker at RT, and acquired using the Bio-Plex® MAGPIX multiplex reader (Bio-Plex Manager,

452   Bio-Rad). The median fluorescence intensity (MFI) for the no-plasma control was subtracted

453   from the MFI for each test sample. Plate variation was normalized using the MFI values

454   measured against each antigen for the standards, the Melbourne naïve controls and positive

455   control, which were used in all plates.

456   **Statistical analysis**

457   Statistical analyses were carried out using PRISM 5 (GraphPad, San Diego CA, USA) and

458   MATLAB R2020a (MathWorks, Natick MA, USA) with the PLS_Toolbox (Eigenvector Research,

459   Inc., Manson WA, USA).

460   In univariate analyses, antibody levels were compared between individuals with severe and

461   uncomplicated malaria and gene transcription was compared between subgroups using

462   Wilcoxon Rank Sum test. Pearson r correlations were determined between log transformed

463   levels of *var* domain transcription (rpkm, reads per kilobase million) and log transformed

464   levels of antibody to the same proteins.

465   Unsupervised machine learning models using MATLAB were used to delineate relationships

466   between the antibody levels measured against the PfEMP1 domains, AMA-1 and CSP in the

467   study groups (56). Thus, signatures of measured features (antibody responses to different

468   PfEMP1 domains) were associated with clinical groupings (severe and uncomplicated

469   malaria). Data was pre-processed through subtraction of the background signal (MFI of beads

470   incubated with no plasma), log-transformation ($y = \log_{10}(x+1)$; x is the data and y is the log-

471   transformed data) and normalization via mean centring and variance scaling of each antibody

472   feature using the z score function in MATLAB.

473 The Least Absolute Shrinkage and Selection Operator (LASSO) penalised logistic regression

474 model was used to identify antibody signatures that best differentiated the two groups (57).

475 Frequency of selected features in resampling (1000 times) was considered as the criterion of

476 variable importance (Figure S2). 10-fold cross validations were performed for each of the

477 resampled datasets.

478 Principal Component Analysis (PCA) was applied to assess the differences between the

479 antibody features identified by LASSO, between the clinical groups. Two-dimensional score

480 plots were generated to visually assess separation between groups using their individual

481 response measurements expressed through the principal components. The percent of

482 variance described by each principal component is a measure of the amount of variance in

483 antibody response.

484 **Ethics approvals**

485 Written, informed consent was obtained from each participant from Papua, Indonesia. Ethical

486 approval was obtained from the Eijkman Institute Research Ethics Commission (project

487 number 46) in Indonesia, Melbourne Health Human Research Ethics committee (project

488 number 2010.284) and Human Research Ethics Committee of the Northern Territory

489 Department of Health & Families and Menzies School of Health Research, Darwin (HREC 2010-

490 1396) in Australia (35).

491

503

504 **Contributions**

505 MFD and SJR conceptualized the study. RNP and NMA designed and oversaw the patient

506 recruitment with RN. Recruitment of participants and oversight of sample collection was done

507 by LT BFS DAH and JM. GT-H, MFD and AP performed the initial bioinformatic analysis. JSR

508 cloned and sequenced PfEMP1 domains, cloned them into the expression vector, and

509 performed the Luminex assay and analysed the Luminex data under the supervision of TD and

510 AWC and drafted the manuscript with SJR and MFD. Proteins were expressed by TT and ET.

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Figure Legends

Figure 1. Levels of antibody to severe malaria associated PfEMP1 domains in Papuans with

severe or uncomplicated malaria. IgG antibody levels to PfEMP1 domains in 28 individuals

with severe malaria (red), 35 individuals with uncomplicated malaria (blue) and nine malaria-

naïve donors (black) are plotted as median fluorescence intensity. Group medians, green

lines. Data compared by Wilcoxon Rank Sum test. Significant associations indicated by p-

values: * < 0.05, ** < 0.01, *** <0.001, **** < 0.0001. Antibody levels were measured against

22 domains upregulated in severe malaria. MFI, median fluorescence intensity; IgG,

immunoglobulin G; UM, uncomplicated malaria; SM, severe malaria; Melb neg, malaria-naïve

negative controls from Melbourne.

Figure 2. Levels of antibody to uncomplicated malaria associated PfEMP1 domains in

Papuans with severe or uncomplicated malaria. IgG antibody levels to PfEMP1 domains in

28 individuals with severe malaria (red), 35 individuals with uncomplicated malaria (blue) and

nine malaria-naïve donors (black) are plotted as median fluorescence intensity. Group

medians, green lines. Data compared by Wilcoxon Rank Sum test. Significant associations

indicated by p-values: * < 0.05, ** < 0.01, *** <0.001, **** < 0.0001. Antibody levels were

measured against 10 domains upregulated in uncomplicated malaria and control antigens

AMA-1, CSP and tetanus toxoid. MFI, median fluorescence intensity; IgG, immunoglobulin G;

UM, uncomplicated malaria; SM, severe malaria; Melb neg, malaria-naïve negative controls

from Melbourne.

741 **Figure 3. Serological signatures of Papuan individuals with severe and uncomplicated**

742 **malaria based on selected antibody features.** A) Principal Component Analysis scores with

743 B) corresponding loadings plot of the three most frequently selected features by LASSO

744 regularised logistic regression of Papuan individuals with severe (n=28; red) and

745 uncomplicated malaria (n=35; blue). The groups were separated across the first principal

746 component (PC) with a variance of 67.81%. Cross validation: venetian blinds with ten splits

747 and blind thickness of one. Data was right-shifted, log-transformed and z scored prior to

748 analysis.

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752 **Tables**

753 **Table 1 Study participants categorized by disease severity.**

Characteristic	Severe malaria (n=28)	Uncomplicated malaria (n=35)
Age (years)	29 [19.5-34]	22.5 [18-25][a]
Female	39.3 (11)	45.7 (16)[a]
Male	60.7 (17)	52.9 (18)[a]
Haemoglobin (g/dL)	11.7 [10.1-13.7]	12.3 [10.6-14.1][a]
Parasite density (parasites/µl)	39400 [8600-252597][b]	27360 [16600-52800][c]

754 Median [interquartile range] or percentage (number). [a] data available for n=34. [b] data

755 available for n=27. [c] data available for n= 28.

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758 **Table 2 Severe malaria syndromes in Papuan individuals with severe malaria.**

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

759 Data presented as percentage (total number). ARDS, acute respiratory distress syndrome

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761

Protein number	Domain expressed	Domain Cassette/ Gene Association
sm1	CIDRα2.4	
sm2	CIDRβ1	
sm3	DBLβ12	DC8
sm4	DBLβ3	
sm5	DBLβ3	DC4
sm6	DBLδ1	
sm8	DBLδ1	
sm9	DBLδ1	DC8
sm11	DBLε3	DC11
sm12	DBLε9	
sm13	DBLεpam5	*Var2*
sm14	DBLγ3	DC9?
sm15	DBLζ4	DC9?
sm17	DBLα1.5	DC4
sm18	CIDRα1.1	DC8
sm19	**CIDRα1.6**	DC4
sm22	DBLε5	*Var1*
sm24	DBLζ3	
sm25	**DBLβ13**	
sm26	CIDRγ12	
sm27	DBLδ7	
sm28	CIDRα2.6/3.4 -DBLβ5/8/13	
um1	DBLα0.13	
um2	**DBLδ1**	
um6	DBLα0.11	
um8	DBLγ9	
um14	DBLδ1	
um19	DBLδ1	
um20	CIDRα3.1	
um21	DBLα0.9	
um43	DBLpam2	
um45	CIDRα1.7	

770
771

A)

Scores on PC 1 (67.81%) vs Scores on PC2 (19.99%)

- uncomplicated malaria (blue)
- severe malaria (red)

B) Loadings Plot

Selected PfEMP1 domains:
- sm19cidra16
- um2dbld1
- sm25dblb13

Loadings on PC1 (67.81%)