Title:

Longitudinal analysis of naturally acquired antibodies to PfEMP1 CIDR domain variants and their association with malaria protection

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ABSTRACT

Malaria pathogenicity is determined, in part, by the adherence of *Plasmodium falciparum* infected erythrocytes to the microvasculature mediated via specific interactions between PfEMP1 variant domains to host endothelial receptors. Naturally acquired antibodies against specific PfEMP1 variants can play an important role in clinical protection against malaria. We evaluated IgG responses against a repertoire of PfEMP1 CIDR domain variants to determine the rate and order of variant-specific antibody acquisition and their association with protection against febrile malaria in a prospective cohort study conducted in an area of intense, seasonal malaria transmission. Using longitudinal data, we found that IgG to the pathogenic domain variants CIDRα1.7 and CIDRα1.8 were acquired the earliest. Furthermore, IgG to CIDRγ3 was associated with reduced prospective risk of febrile malaria and recurrent malaria episodes. Future studies will need to validate these findings in other transmission settings and determine the functional activity of these naturally acquired CIDR variant-specific antibodies.

53 **INTRODUCTION**

54 Malaria due to *Plasmodium falciparum* causes greater than 400,000 deaths per annum (1).
55 Severe clinical manifestations of *P. falciparum* malaria are precipitated by widespread
56 sequestration of infected erythrocytes (IEs) in host microvasculature including in the brain and
57 placenta which can lead to cerebral malaria and placental malaria, respectively (2).
58 Cytoadherence of IEs occurs via specific interactions between host endothelial receptors and *P.*
59 *falciparum* erythrocyte membrane protein (PfEMP1), a parasite-derived protein expressed on the
60 surface of IEs that is a major target of naturally acquired immunity to malaria (3-5). The PfEMP1
61 adhesins are encoded by ~60 *var* gene variants that differ within and between parasite genomes
62 and that are expressed in a mutually exclusive manner within each IE (6-8). Switching between
63 *var* genes aids in parasite immune evasion and functional diversification of the PfEMP1 family
64 have resulted in mutually exclusive receptor binding phenotypes correlated to differences in
65 clinical severity (9, 10).

66 Members of the PfEMP1 family vary in the size and number of extracellular Duffy-binding-
67 like (DBL) and cysteine-rich interdomain region (CIDR) domains(11). DBL and CIDR domains are
68 classified based on sequence similarity into six (α, β, γ, δ, ε, ξ) and four (α, β, γ, δ) main classes,
69 respectively, of which some can be further divided into sub-classes (e.g. CIDRα1.1) (12, 13).
70 PfEMP1 generally have a semi-conserved head structure near the N-terminus consisting of a
71 tandem DBLα-CIDR domain. This can be followed by a second DBLδ-CIDR tandem domain or
72 additional other types of DBL domains in larger proteins. Notably, however, the VAR2CSA PfEMP1
73 variants do not contain typical CIDR domains and bind placental chondroitin sulfate A via
74 specialized DBL domains (14, 15). PfEMP1 have diversified to either bind endothelial protein C

75 receptor (EPCR) (10), the scavenger receptor CD36 (16) or yet undermined receptors via their
76 head structure CIDR domains. These phenotypes are maintained by the chromosomal
77 organization of the *var* genes (17). Among the subtelomeric *var* genes, Group A genes transcribed
78 toward the telomere encode DBLα1-CIDRα1 head structures binding to EPCR or DBLα1-
79 CIDRβ/γ/δ head structures with unknown endothelial receptor specificities. Subtelomeric Group
80 B *var* genes transcribed toward the centromere as well as centromeric Group C *var* genes encode
81 DBLα0-CIDRα2-6 head-structures binding to CD36. In addition to this, chimeric group B/A *var*
82 genes encode EPCR-binding DBLα0-CIDRα1 head structures. The EPCR-binding phenotype has
83 been implicated in severe malaria (18-21), whereas CD36 binding has been associated with
84 uncomplicated malaria (22, 23). Severe malaria has been associated with rosetting, a
85 phenomenon which involves binding between an IE and several uninfected erythrocytes but with
86 unclear clinical significance. A set of group A PfEMP1 with DBLα1-CIDRβ/γ/δ domains have been
87 shown to mediate rosettes.

88 Immunity to severe malaria is generally acquired after only one to two severe episodes (24)
89 with naturally acquired antibodies specific for PfEMP1 variants likely playing an important role in
90 clinical protection (25). Antibodies to group A PfEMP1 variants tend to be acquired prior to
91 antibodies to group B and C variants (26) and are associated with protection from severe malaria
92 (27). Similarly, antibodies to EPCR-binding CIDRα1 domains are acquired more rapidly than
93 antibodies to other CIDR domains in areas of high malaria transmission intensity and are boosted
94 by severe malaria but not uncomplicated malaria (28, 29). However, a recent study showed that
95 antibodies to both rosetting-associated DBLα variants and CD36-binding CIDR domains predicted
96 reduced risk of severe malaria to a similar extent as antibodies to EPCR-binding CIDR domains

97 (30). The same study also showed that antibodies to group 2 DBLα variants, which are associated
98 with rosetting (31), also predicted protection from uncomplicated malaria.

99 To gain further insight into the role of PfEMP1-variant specific antibodies, we assessed IgG
100 responses against a repertoire of PfEMP1 CIDR domains to determine the rate and order of
101 variant-specific antibody acquisition and their association with protection against uncomplicated
102 febrile malaria in a prospective cohort study conducted in a Malian village with intense and
103 seasonal malaria transmission.

104 **RESULTS**

105 **IgG specific for CIDRα1, CIDRδ, and CIDRγ domain variants are acquired rapidly.**

106 Naturally acquired IgG antibody responses to 35 PfEMP1 CIDR domain variants representing
107 subtypes α, γ and δ CIDR, as well as three well-studied *P. falciparum* antigens (circumsporozoite
108 protein [PfCSP], apical membrane protein 1 [PfAMA1], and merozoite surface protein 1
109 [PfMSP1]), tetanus toxoid (non-malaria positive control), and bovine serum albumin (non-specific
110 background control; Table S1) were determined by multiplex bead-based immunoassay in 680
111 children and adults from the Kalifabougou, Mali cohort at their healthy baseline in May 2011 (Fig.
112 S1). Hierarchical clustering of baseline PfEMP1-specific IgG reactivity revealed distinct clustering
113 of samples by age, and by the presence of PCR-documented, asymptomatic *P. falciparum*
114 infection; as well as clustering of antigen targets by group (A, B, or B/A), binding phenotype
115 (EPCR, CD36, or unknown), and CIDR domain class (Fig. 1a); suggesting differential rates of
116 acquisition of IgG between PfEMP1 variants with cumulative *P. falciparum* exposure and the
117 acquisition of clinical immunity to malaria. PfEMP1-specific IgG reactivity increased rapidly up to

118 8 years of age, and within each age stratum, *P. falciparum* PCR-positive individuals exhibited
119 greater variant-specific IgG reactivity than uninfected individuals (Fig. 1b).
120 Categorization of PfEMP-1 variants by CIDR domain class suggested that IgG specific for
121 variants in the CIDRγ, CIDRα1, and CIDRδ classes was acquired rapidly whereas IgG specific for
122 group B variants of the CIDRα2-6 class was acquired slowly irrespective of *P. falciparum* infection
123 status (Fig. 1c). Indeed, when compared to variants of other domain classes within the linear
124 range of the fit curves (<8 years of age), IgG specific for variants within each of the CIDRγ, CIDRα1,
125 and CIDRδ classes increased significantly more rapidly with age, whereas IgG specific for variants
126 of the CIDRα2-6 classes increased significantly more slowly with age, independent of *P.*
127 *falciparum* infection status (Fig. 1d and Table S2). Of note, IgG specific for AMA1, CSP, and MSP1
128 increased predictably with age in early childhood and plateaued in adolescence or young
129 adulthood, which is similar to what we previously observed in this cohort (32, 33) (Fig. 1b-c). As
130 we observed previously by ELISA in a separate cohort in Mali (34), increases in tetanus toxoid-
131 specific IgG in early childhood and adolescence corresponded with the primary childhood vaccine
132 series (diphtheria, tetanus, pertussis) and a subsequent booster of a tetanus toxoid-containing
133 vaccine in females of child-bearing age (Fig. 1b-c).
134 With the exclusion of children <6 months of age whose IgG is most likely maternally derived,
135 ranking of antigens by decreasing seropositivity within each age group revealed
136 immunodominance of CIDRα1, CIDRδ, and CIDRγ domain classes, which are all either of the A or
137 B/A *var* group, in early childhood (<7 years) that is maintained to a large degree in adolescence
138 and early adulthood (Fig. S2). Notably, the most prevalent PfEMP1-specific IgG reactivity among
139 individuals greater than 1 year of age was against CIDRα1.7(c) with seroprevalence rapidly rising

140 from 25% in 2 to 3-year-old children to 60% in 4 to 6-year-old children and surpassing 95% in
141 older children and adults (Fig. S2). However, the majority of individuals within the oldest age
142 group (15-25 years) were also seropositive for several variants within the CIDRα2-6 domain
143 classes, suggesting that IgG antibodies against these variants are eventually acquired with
144 additional years of malaria exposure.

145 To assess the longitudinal acquisition of variant-specific IgG, we determined variant-specific
146 IgG reactivity across five annual cross-sectional surveys conducted just prior to each malaria
147 transmission season for an age-stratified random sample of 60 children from the entire cohort
148 (Fig. S1). Children in this subset experienced a median of 6 febrile malaria episodes (interquartile
149 range, 4–9 episodes) with a broad range of parasite densities and distributed widely but with
150 clear seasonal peaks in the number of episodes during the five-year surveillance period (Fig. 2a-
151 b). In the youngest children (6 months to 2 years), IgG specific for variants of the CIDRα1 and
152 CIDRδ domain classes began low and then increased rapidly over four malaria seasons, whereas
153 IgG specific for CIDRγ initially decreased during the first two years before rising during the third
154 year of surveillance (Fig. 2c). In contrast, older children (3 to 8 years) appeared to maintain stable
155 levels of IgG specific for all PfEMP1 variants over four malaria seasons (Fig. 2c).

156 **Acquisition of IgG antibodies to CIDR domain classes is highly ordered with IgG against EPCR-**
157 **binding domain variants CIDRα1.7 and CIDRα1.8 acquired first.**

158 We next asked whether IgG antibodies to individual PfEMP1 variants were acquired in a particular
159 order. Here we used an approach called *minimum violations ranking* (MVR), where an algorithm
160 searches over different possible orders of acquisition of antibodies to PfEMP1 variants such that,
161 if a particular an order is assumed for each child, the number of order violations observed in the

162 data overall is minimized (refer to methods). We observed significantly less violations if we
163 assumed an ordered acquisition of antibodies compared to a model with randomized
164 seroconversion orders for each child, which highly suggests a hierarchical exposure to different
165 parasite CIDR domains in this population (Fig. 3a-d). At the variant level, IgG specific to
166 CIDRα1.7(c) was acquired first followed by IgG to CIDRα1.8b(a), CIDRα1.8b(c), CIDRα1.7(a),
167 CIDRα6, and CIDRγ3 (Fig. 3a). Grouped by CIDR domain class, IgG was acquired against CIDRγ first
168 followed by CIDRα1, CIDRδ, and CIDRα2-6 (Fig. 3b). Grouped on the basis of upstream sequence,
169 IgG was acquired against B/A first followed by A and B (Fig. 3c). Lastly, when variants were
170 grouped by binding phenotype, IgG against EPCR-binding domains were acquired first followed
171 by domains with unknown binding phenotypes and CD36-binding domains (Fig. 3d). Whether
172 this reflects differential prevalence of variants in the parasite population or age-specific
173 expression patterns remains an open question.

174 **CIDRγ-specific IgG associates with protection from uncomplicated, febrile malaria.**

175 We focused on the risk of uncomplicated malaria given that severe malaria was rarely observed
176 in the Kalifabougou cohort due to early diagnosis and treatment. We specifically evaluated
177 whether baseline seropositivity for each variant could predict protection from febrile malaria
178 after subsequent PCR-confirmed *P. falciparum* parasitemia in individuals who began the study
179 PCR-negative using a Cox regression model that included age, presence of the malaria-protective
180 HbS allele, gender, IgG reactivity to AMA1 (as a surrogate for prior malaria exposure), and
181 seropositivity to each of the 35 PfEMP-1 variants as covariates. Notably, seropositivity to CIDRγ3
182 (IT4var08), which has an unknown binding phenotype was significantly associated with reduced
183 risk of febrile malaria (Table 1). CIDRγ domains have been associated with rosetting of

184 erythrocytes (11), a phenomenon associated with severe forms of malaria (35) except in
185 individuals with blood group O erythrocytes which appear to exhibit reduced rosetting (36). We
186 therefore hypothesized that the reduced risk afforded by CIDRγ-specific IgG might occur via the
187 inhibition of rosette formation and may therefore be negatively affected by blood group O. When
188 included as a covariate in a reduced Cox regression model, group O blood type affected neither
189 malaria risk itself nor the association between CIDRγ-specific IgG and risk of febrile malaria (Table
190 S3). Notably, baseline CIDRγ3-specific IgG reactivity did not significantly correlate with decreased
191 parasite density at the first malaria episode after controlling for age and the presence of the HbS
192 allele (data not shown), suggesting that CIDRγ-specific IgG may not have anti-parasite activity.
193 Given the association between CIDRγ-specific IgG and delay in malaria fever during the first year
194 of the study, we specifically examined if CIDRγ3 serostatus at the beginning of each malaria
195 season affected the risk of recurrent malaria episodes in the 60 children who were longitudinal
196 evaluated for PfEMP1 IgG responses over five malaria seasons. Presence of CIDRγ3-specific IgG
197 prior to each season predicted a reduction in febrile malaria episodes even after controlling for
198 AMA1-specific IgG serostatus and the HbS allele (Table 2).

199 **DISCUSSION**

200 PfEMP1 variants containing domains of the CIDRα1 class generally bind to EPCR on
201 endothelial cells and are associated with severe malaria (10), whereas variants containing
202 domains of the CIDRα2-6 classes bind to CD36 present on several host cell types, including
203 microvascular endothelial cells, mononuclear phagocytes, and platelets (16, 37). Antibodies
204 targeting these PfEMP1 domains can potentially disrupt adhesion of IEs to host receptors but can
205 also facilitate IE clearance via opsonization and phagocytosis or antibody-mediated cytotoxicity

206 (10, 38, 39). Consistent with a prior study conducted in a Tanzanian cohort (28), we observed
207 early acquisition of IgG antibodies against EPCR-binding PfEMP1 variants of the CIDRα1 domain
208 class relative to CD36-binding variants in both age-stratified cross-sectional and longitudinal
209 analyses. This is also consistent with studies that investigated acquisition of antibodies to PfEMP1
210 classified by upstream sequence group and found that antibodies to DBL and CIDR domains
211 belonging to group A and B/A are acquired earlier in life than group B and C variants among
212 individuals living in malaria-endemic settings (26, 40). Importantly, the antigen panel used in the
213 current study contained unique CIDR domains not covered by these prior studies.

214 Among the 35 distinct CIDR domains evaluated here, CIDRα1.7(c) elicited the most robust
215 and prevalent IgG responses in early childhood, eventually approaching 100% seroprevalence in
216 adolescents and adults in this cohort. Longitudinal analysis to assess hierarchical acquisition
217 confirmed that IgG antibodies specific for CIDRα1.7(c) were acquired first, with IgG against the
218 related CIDRα1.7(a) variant acquired fourth. Transcripts encoding CIDRα1.7 domains have been
219 found to predominate among the most severe cases of pediatric cerebral malaria—those that
220 lead to brain swelling and death (19). The immunodominance of CIDRα1.7(c) may be a
221 consequence of epitopes targeted by cross-reactive CIDRα1 antibodies (41, 42). Moreover,
222 PfEMP1 with CIDRα1.4 and CIDRα1.7 domains frequently contain ICAM1-binding DBLβ domains
223 (43). The dual EPCR- and ICAM1-binding phenotype is thought to be particularly pathogenic, and
224 antibodies to these DBLβ domains have been associated with reduced risk of clinical malaria with
225 parasite densities of ≥10,000 parasites/μl (44). We also observed early acquisition of IgG specific
226 for CIDRα1.8 domains. Expression of these domains, as well as EPCR-binding CIDRα1 domains in

227 general, is associated with severe malaria including cerebral malaria in African children (18-21,
228 45) and Indian adults (46).

229 Given that all CIDRα1 variants have been linked to severe malaria in African children, the early
230 acquisition of IgG specific to CIDRα1.7 and CIDRα1.8 domains may just be a reflection of local
231 parasite population dynamics rather than enhanced pathogenicity conferred by these specific
232 CIDR variants. However, the potential lethality of parasites expressing CIDRα1 in general
233 underscores why a vigorous host antibody response against these variant domains in early
234 childhood may be advantageous. This study builds on older work (4, 47, 48) showing an age-
235 specific acquisition of antibodies to particular parasite strains, and we are able to statistically
236 confirm this pattern for the first time, and identify key genetic underpinnings of those
237 observations. We still cannot address the slippery problem of whether this order reflects the
238 circulation of genotypes with different transmissibility; under this scenario, high fitness
239 genotypes lead to high prevalence and therefore low age of first infection, and coincidentally
240 cause more disease in relatively non-immune children compared to low fitness genotypes as a
241 result. In contrast, it is possible that the ordered expression of PfEMP1 variants across strains,
242 potentially in response to the immune status of the parasite's immediate host, leads to the
243 hierarchical acquisition of antibodies observed.

244 Due to the low incidence of severe disease in the cohort, we could not assess the impact of
245 CIDRα1.7-specific or CIDRα1.8-specific antibodies on the risk of severe malaria in the study.
246 However, when all 35 CIDRs were assessed for association with the prospective risk of
247 uncomplicated, febrile malaria, IgG specific to CIDRγ3 (IT4var08) was significantly associated with
248 reduced malaria risk. PfEMP1 variants encoding CIDRβ, CIDRγ, or CIDRδ domains have been

249 associated with rosetting (11, 46), which can enhance microvasculature obstruction thereby
250 increasing malaria severity. However, direct evidence that any of these CIDR domains have
251 intrinsic rosetting properties is lacking (49). Rather, their association with rosetting may be
252 related to their tandem expression with an adjacent DBLα1 at the N-terminal head (50). Rosetting
253 frequency has been correlated with severity of malaria with the highest levels in cerebral malaria
254 (35, 51, 52) but is still commonly observed in uncomplicated malaria. Thus, the role of rosetting
255 in severity of malarial disease remains unclear. Nevertheless, disruption of rosettes by targeting
256 DBL1α has been used as a vaccine strategy (53), and antibodies to rosetting-associated group 2
257 DBLα domains predicted protection from uncomplicated malaria, suggesting a protective role for
258 these antibodies in less severe disease (30, 31). Although speculative, it is possible that naturally
259 acquired CIDRγ-specific IgG confers protection from febrile malaria by blocking rosette
260 formation. However, this mechanism is not supported by the current study given that the
261 protection attributable to CIDRγ-specific IgG is unchanged after controlling for blood group O,
262 which has been shown to be protective against severe falciparum malaria through the reduction
263 of rosetting (36). It also must be noted that reduced malaria risk was not observed for IgG-specific
264 for variants of the CIDRδ class, which is also predicted to have rosetting activity. Furthermore, as
265 CIDRγ3 was the only CIDRγ domain variant tested in this study, it remains unknown whether the
266 protective effect observed here would be generalizable to IgG targeting other CIDRγ variants.

267 A limitation of the study is that we did not sequence *var* transcripts from individuals with *P.*
268 *falciparum* infections in the longitudinal analysis. This may have allowed us to prospectively
269 assess if seroconversion against specific CIDRs such as CIDRα1.7, CIDRα1.8, or CIDRγ reliably led
270 to the absence of parasites expressing the corresponding *var* transcript during clinical malaria

271 episodes. In addition to our limited assessment of CIDRγ domains, we also did not evaluate CIDRβ
272 domains, which also have been associated with the rosetting phenotype.

273 In summary, this longitudinal study provides evidence that acquisition of IgG antibodies to
274 PfEMP1 variants is ordered and demonstrates that antibodies to CIDRα1 domains, specifically the
275 pathogenic domain variants CIDRα1.7 and CIDRα1.8, are acquired the earliest in children residing
276 in an area of intense, seasonal malaria transmission. We also show that IgG antibodies to the
277 rosetting-associated CIDRγ3 domain is acquired early and is associated with protection from
278 febrile malaria. Future studies will need to validate these findings in other transmission settings
279 and determine the functional activity of these naturally acquired CIDR variant-specific antibodies.

280 **METHODS**

281 **Ethics**

282 The Ethics Committee of the Faculty of Medicine, Pharmacy and Dentistry at the University
283 of Sciences, Techniques, and Technology of Bamako, and the Institutional Review Board of the
284 National Institute of Allergy and Infectious Diseases, National Institutes of Health approved this
285 study (ClinicalTrials.gov identifier: NCT01322581). Written, informed consent was obtained from
286 the parents or guardians of participating children or from adult participants.

287 **Study Site**

288 The study was conducted in the village of Kalifabougou, Mali, which is located 40 km
289 northwest of Bamako, Mali within the savanna ecoclimatic zone. Within this community,
290 Bambara is the predominant ethnic group, and ~90% of residents engage in subsistence farming.
291 Malaria transmission is intense and seasonal, reliably occurring from June through December,
292 with the vast majority of malaria cases caused by *P. falciparum* (54).

293 **Study Population and Study Design**

294 Recruitment and enrollment procedures of participants for this study have been previously
295 described (55). Briefly, exclusion criteria at enrollment included a hemoglobin level <7 g/dL,
296 axillary temperature ≥37.5°C, acute systemic illness, underlying chronic disease, use of
297 antimalarial or immunosuppressive medications in the past 30 days, or pregnancy. The study
298 design and selection of subjects are summarized in Fig. S1.

299 **Human samples**

300 At the beginning and end of the malaria-transmission season, blood samples were drawn by
301 venipuncture into sodium-citrate-containing Vacutainer tubes (Becton Dickinson). Plasma was
302 separated by centrifugation and cryopreserved. Hemoglobin typing was performed using a D-10
303 instrument (Bio-Rad). Blood for ABO typing was collected in EDTA containing microtainers. ABO
304 typing was conducted with forward typing using Cypress Diagnostics Reagents. Anti-A, Anti-B,
305 and Anti-AB IgM reagents were mixed with the sample, and blood type was determined by
306 agglutination. During the first malaria season, blood was collected by finger-prick onto 903 filter
307 paper (Whatman) for PCR analysis at each scheduled clinic visit (occurring at 2-week intervals for
308 7 months) and sick visit for subsequent molecular diagnostics.

309 **Diagnosis and Treatment of Infections**

310 **Clinical malaria episodes.** Individuals were initially enrolled in May 2011 and have been
311 followed continuously since unless withdrawn or lost to follow-up. During the first malaria
312 season, clinical malaria episodes were detected prospectively by self-referral and weekly active
313 clinical surveillance visits which alternated between the study clinic and the participants' homes.
314 Passive malaria surveillance and pre- and post-malaria season cross-sectional surveys have

315 continued during subsequent years. All individuals with signs and symptoms of malaria and any
316 level of *Plasmodium* parasitemia detected by light microscopy were treated according to the
317 National Malaria Control Program guidelines in Mali. For the current study, a clinical malaria
318 episode was defined as any parasitemia on contemporaneous blood smear, an axillary
319 temperature of ≥37.5°C within 24 hours, and no other cause of fever discernible by physical
320 exam.

321 **Blood smears.** Thick blood smears were stained with Giemsa and counted against 300
322 leukocytes. Parasite densities were recorded as the number of asexual parasites/µl of blood
323 based on a mean leukocyte count of 7500 cells/µl. Each smear was read in blinded manner by
324 two certified microscopists of the laboratory team.

325 **Molecular detection**. For each participant, the first *P. falciparum* infection of the initial
326 malaria season was detected retrospectively by PCR analysis of the longitudinally collected dried
327 blood spots (54). First malaria episodes were determined from the clinical visit data.

328 **Protein Expression and Multiplex Immunoassays**

329 The 35 recombinant His-tagged CIDR domains (Table S1) were expressed in baculovirus-
330 transfected insect cells, and purified by nickel affinity chromatography as previously described
331 (28, 42, 56). AMA1, CSP, and MSP1 recombinant proteins were kindly provided by David Narum
332 (Laboratory of Malaria Immunology and Vaccinology, NIAID, NIH). AMA1 and CSP were expressed
333 from *P. falciparum* 3D7 in *P. pastoris* as previously described (57, 58). MSP1 was expressed from
334 *P. falciparum* 3D7 in *Escherichia coli* as previously described (59). Purified tetanus toxoid was
335 provided the staff at Biologic Laboratories, University of Massachusetts Medical School. Bovine
336 serum albumin (BSA) was obtained from Sigma. These proteins were coupled to MagPlex-C

337 micospheres (Luminex) and mixed to form a protein bead array in which IgG reactivity to each
338 antigen could be measured in multiplex, as previously described(60) with minor modifications.
339 Briefly, plasma samples were diluted 1:500 and 1:2000 (to better assess highly reactive antigens)
340 in Assay Buffer E (ABE: 0.1% BSA, 0.05% Tween-20 in PBS, pH7.4). For each plate, pooled malaria-
341 hyperimmune plasma was serially diluted in ABE at 1:50, 1:158, 1:500, 1:1580, 1:5000, 1:1580,
342 1:50000, and 1:158000 to generate an 8-point dilutional standard curve. 50 µl of beads and 50 µl
343 of diluted plasma was added to 96-well microtiter plates (MSBVS 1210, Millipore, USA) pre-
344 wetted with ABE. 50 µl of phycoerythrin-conjugated Goat Anti-Human IgG (Jackson
345 ImmunoResearch Laboratories), diluted 1:3000 was added, and mean fluorescent intensities
346 were measured using the Luminex 200 system. To account for plate-to-plate variation,
347 fluorescence intensities were normalized using the median reactivity for each antigen on each
348 plate. Normalized intensities were then scaled to the mean reactivity for each antigen to allow
349 comparison between antigens. Using the ncal function within the nCal package(61), IgG
350 concentrations were interpolated from the standard curves generated from serial diluted pooled
351 malaria-immune plasma and the resulting concentrations reported as arbitrary units (AU), which
352 was used for statistical analyses and visualization.

353 **Ordered Acquisition Analysis**

354 If seroconversion to CIDR domains occurs in a stereotypical order, then each individual's
355 sequence of seroconversions in this longitudinal study should be congruent with that order. Of
356 course, we do not know such an order *a priori*, so we find it by searching over all orderings to
357 find the one that minimizes the number of order-violating seroconversions. This *minimum*
358 *violations ranking* (MVR) consists of both the ordinal ranking itself and a corresponding number

359 of rank violations *v*. These outputs can be visualized by plotting a heatmap, with indices ordered
360 by the minimizing ranking as in Fig. 3A. Clear triangular structure indicates the strength of the
361 ordering, and *v* is equal to the sum of the sparser triangle.

362 Note that the more that individuals' seroconversions occur strictly in their rank order, the
363 smaller *v* will be. In this way, the number of violations *v* provides a convenient test statistic for a
364 standard one-tailed p-value test: our null hypothesis is that there is no meaningful order to
365 seroconversions, and thus, randomly permuting the order of seroconversions for each individual
366 and recomputing *v* should make no difference. In other words, the null hypothesis is that the
367 number of violations *v* in the real data is statistically indistinguishable from the number of
368 violations in the time-randomized data v_{random}. The p-value can be computed then as $p=\Pr(v <$
369 $v_{random})$. When actual seroconversions are significantly more orderable than random
370 seroconversions (while preserving the seroconversion counts per individual and seroconversions
371 per CIDR domain), it indicates the presence of a statistically significant stereotypical ordering, as
372 in Fig. 3B.

373 Computations were performed according to the following details. Let matrix entry A_{ij} be the
374 number of times, over each individual, that a seroconversion to i was observed prior to a
375 seroconversion to j. If the matrix's rows and columns are sorted according to some re-ordering r,
376 then the number of violations v can be computed as the sum of the lower triangle of A(r). Finding
377 the r that minimizes v can be done by beginning from a random r, and then sequentially proposing
378 swaps of pairs of indices in which any swap that increases v is rejected and otherwise swaps are
379 accepted. This MVR algorithm exits after a large number of proposed swaps have been rejected
380 without any decrease in v, and the output is both v and the order of seroconversion that

381 corresponds to that v. Permutation tests were then performed by shuffling the seroconversions
382 and years, independently for each individual, and then applying the computation above.

383 **Statistical Analysis**

384 The use of specific statistical tests and methods are indicated in the Results and/or figure
385 legends. Statistical significance was defined as a 2-tailed P value of <.05. Analyses were
386 performed in R version 3.6.1 (http://www.R-project.org). Plots were generated with the *ggplot2*
387 package. Cox regression was performed using the *survival* and *glmnet* packages. For the time to
388 febrile malaria analysis (Table 1), variable selection from among the 35 CIDR seropositivity
389 variables, age, gender, AMA1 seroreactivity, and the presence of the HbS allele was determined
390 using regularized Cox regression fit with the least absolute shrinkage and selection operator
391 (LASSO) penalty using 10-fold cross validation with 1000 iterations (62). The follow-up period
392 after initial blood-stage infection was 60 days. For the final Cox regression model, age, gender,
393 and the presence of the HbS allele were included as co-variates along with the LASSO-selected
394 CIDR variables. For the recurrent event analysis, the Andersen-Gill extension of the Cox
395 regression model was used to determine the relative risk of malaria over five malaria seasons
396 using presence of the HbS allele as a covariate and AMA1-specific IgG seropositivity (a surrogate
397 for overall malaria exposure) and CIDRγ3-specific IgG seropositivity as time-dependent covariates
398 that varied over each season.

399

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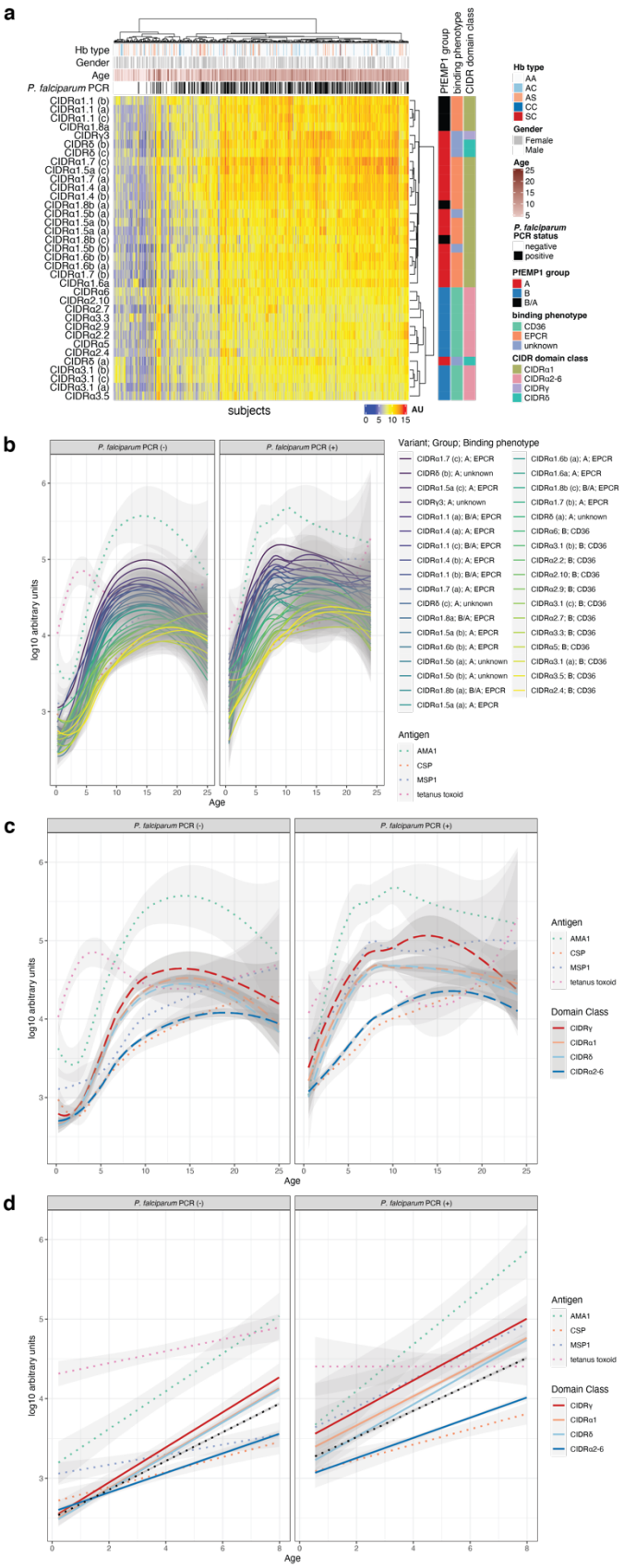
413 **Author Contributions**

414 NO, LHM, SKP, LT, TL, PDC, and TMT conceived the study. AO, SD, KK, and BT were responsible
415 for the cohort study and collection of samples. NO, LT, SL, and TBY conducted the experiments.
416 NO, DBL, and TMT analyzed the data. TMT, TL, CB, DBL, and PDC wrote the manuscript with
417 contributions from NO, LT, LHM, and SKP. All authors read and approved the manuscript.

Fig. 1. IgG antibodies to PfEMP-1 variants belonging to the A or B/A groups or having the EPCR-binding phenotype are rapidly acquired during childhood.

a Hierarchical clustering heatmap showing IgG reactivity to each of the 35 PfEMP-1 variants in 680 subjects at enrollment (May 2011 healthy baseline). Clustering was performed using the Ward.D method and the Pearson distance metric. AU refers to arbitrary concentration units, which was calculated by fitting data to a dilutional standard curve of pooled hyper-immune plasma from malaria-exposed Malian adults. **b-c** IgG reactivity obtained at May 2011 healthy baseline versus age for each PfEMP-1 variant (solid lines) or grouped by CIDR domain class (dashed lines) with loess fit curves and 95% confidence intervals. Control antigens shown as dotted colored lines. **d** Linear portion of plot in **b** (age range 3 months to 8 years) with linear fit curves and 95% confidence intervals (see Table S2). For comparison, regression line for all variants together is represented by the black dotted line.



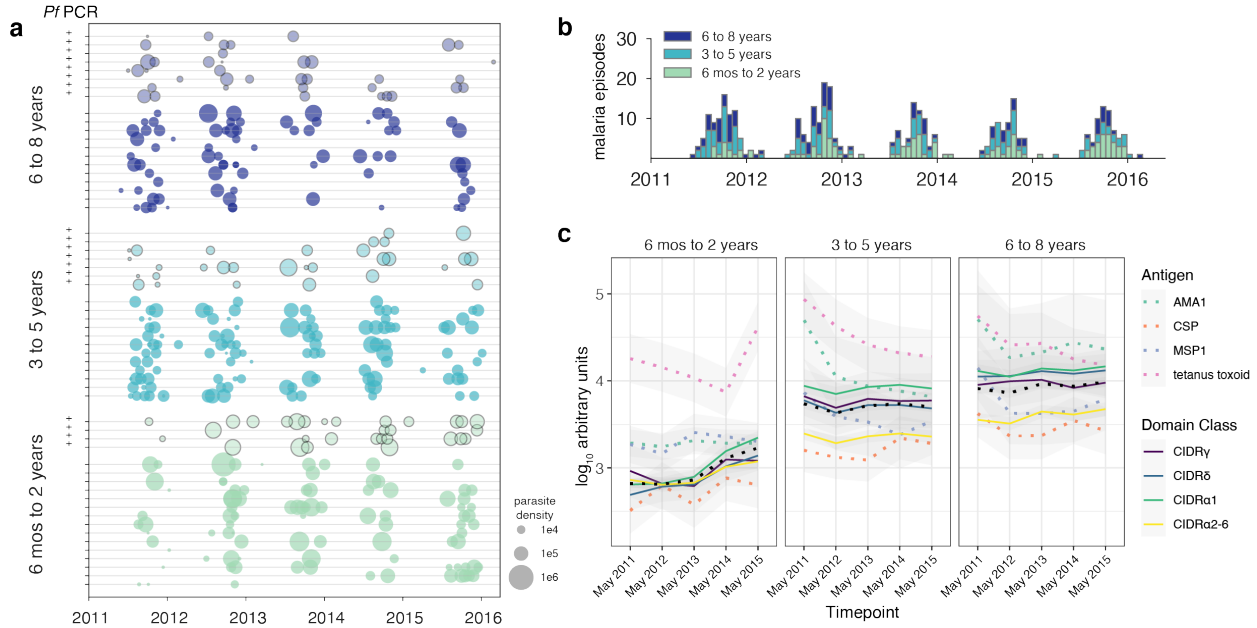
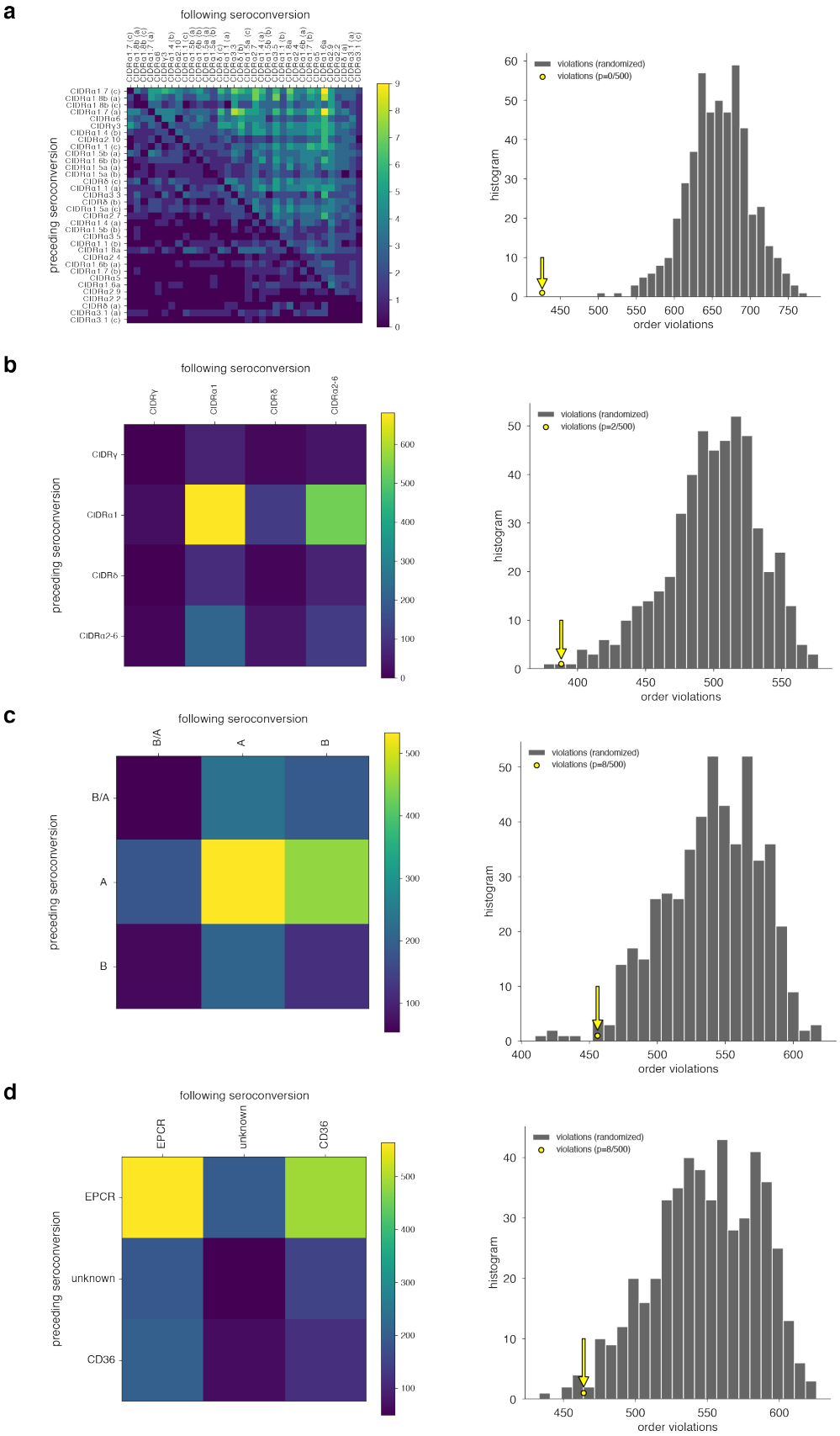


Fig. 2. Longitudinal analysis of PfEMP-1 variant-specific IgG over multiple malaria seasons.

IgG reactivity specific to PfEMP-1 variants was determined for 60 children ages 6 months to 8 years (**see Figure S1**) at five cross-sectional surveys prior to the malaria season. **a** Malaria incidence over five malaria seasons for 60 children in children aged 6 months to 2 years; 3 to 5 years; and 6 to 8 years (n = 20 per age group). Plus (+) signs in the left margin indicate subjects with asymptomatic *P. falciparum* parasitemia at enrollment. Size of bubble is proportional to parasite density determined at each visit. **b** Number of malaria episodes per two-week period by age group over five malaria seasons. **c** Longitudinal IgG reactivity at five cross-sectional surveys in the same children. Color scale for variant is ordered by slopes estimated from cross-sectional data (**Table S2**) to facilitate comparison.


a
following seroconversion
preceding seroconversion
violations (randomized)
violations (p=0/500)
histogram
order violations
b
following seroconversion
CIDRγ
CIDRα1
CIDRδ
CIDRα2-6
preceding seroconversion
violations (randomized)
violations (p=2/500)
histogram
order violations
c
following seroconversion
B/A
A
B
preceding seroconversion
violations (randomized)
violations (p=8/500)
histogram
order violations
d
following seroconversion
EPCR
unknown
CD36
preceding seroconversion
violations (randomized)
violations (p=8/500)
histogram
order violations

430

431 **Fig. 3. Acquisition of IgG to PfEMP-1 variants over time is hierarchical.**

432 Using longitudinal data, seropositivity was determined for each variant within each subject at each time
433 point to determine the year of seroconversion. Seroconversion year was then used to generate a matrix
434 representing the number of times that seroconversion for a variant (rows) precedes another variant
435 (column) across all subjects. To find consensus ordering, the matrix was sorted to minimize the number
436 of violations. Observed consensus ordering was compared against 200 independent procedures in which
437 the seroconversion orders for each subject was randomized and consensus ordering was carry out in the
438 same manner (right panel). Analysis was performed at the level of **a** individual variants, **b** CIDR domain
439 class, **c** upstream sequence group, and **d** binding phenotype.

440 **Table 1. Relationship between CIDR variant seropositivity and protection from febrile malaria.**
441

Variant	Number seropositive (N=271)	Alternate name	PfEMP1 group	Binding Phenotype	Genome/ Isolate	HR	LCI	UCI	P value
CIDRγ3	129	IT4var08	A	unknown	IT4	0.607	0.42	0.876	0.00763
CIDRα3.1 (a)	17	DD2var01	B	CD36	DD2	0.208	0.0523	0.828	0.0259
CIDRα3.3	55	IT4var26	B	CD36	IT4	0.806	0.458	1.42	0.454
CIDRα2.10	37	IT4var30	B	CD36	IT4	0.861	0.507	1.46	0.58
CIDRα2.9	25	IT4var45	B	CD36	IT4	0.83	0.429	1.6	0.58

442
443 Results of Cox regression model assessing PfEMP1 variant-specific IgG on the risk of febrile malaria after
444 incident *P. falciparum* infection in which covariates were age, gender, presence of the HbS allele, and IgG
445 seropositivity for five CIDR variants selected using the least absolute shrinkage and selection operator
446 (LASSO; refer to Methods). Analysis was restricted to subjects who were at least 6 months of age and
447 began the study negative for *P. falciparum* infection by PCR (271 subjects). Malaria risk was determined
448 based on time to clinical malaria, defined as axillary temperature >37.5 degrees C and any parasitemia,
449 after PCR-documented blood-stage infection (163 malaria events). Follow-up time was limited to 60 days
450 from initial blood-stage infection. Results are ordered by increasing P values. HR = hazard ratio; LCI = lower
451 95% confidence interval; UCI = upper 95% confidence interval.

452 **Table 2. Relationship between CIDRγ3 seropositivity and protection from recurrent malaria episodes.**
453

Variable	RR	LCI	UCI	P value
CIDRγ3 seropositive	0.652	0.486	0.875	0.00433
AMA1 seropositive	1.14	0.862	1.51	0.355
HbS allele	0.437	0.309	0.619	2.99E-06

454
455 Results of the Andersen-Gill extension of the Cox regression model to assess the relationship between
456 CIDRγ3-specific IgG seropositivity and the risk of recurrent febrile malaria episodes (defined as fever
457 >37.5°C and any parasitemia; 376 events) in 60 children who were followed longitudinally over five
458 malaria transmission seasons from 2011 through 2015. Presence of the HbS allele and AMA1
459 seropositivity, a surrogate for overall malaria exposure, were included as covariates. CIDRγ3-specific and
460 AMA1-specific IgG seropositivity were treated as time-dependent covariates that varied over each season.
461 RR = relative risk; LCI = lower 95% confidence interval; UCI = upper 95% confidence interval.

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