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Naturally acquired antibody responses to recombinant Pfs230 and Pfs48/45 transmission blocking vaccine candidates.

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33

34 **Abstract.**

35 Objectives: Pfs48/45 and Pfs230 are *P. falciparum* sexual stage proteins and
36 promising malaria transmission-blocking vaccine candidates. Antibody responses
37 against these proteins may be naturally acquired and target antigens may be under
38 selective pressure. This has consequences for the future evaluation of vaccine
39 immunogenicity and efficacy in populations naturally exposed to malaria.

40 Methods: We determined naturally acquired antibody responses to the recombinant
41 proteins Pfs48/45 -10C and Pfs230-230CMB in children from three malaria endemic
42 settings in Ghana, Tanzania and Burkina Faso. We also examined genetic
43 polymorphisms in the *P. falciparum* gene *pfs48/45*.

44 Results: Antibody prevalence was 1.1-18.2% for 10C and 6.7-18.9% for 230CMB. In
45 Burkina Faso we observed evidence of an age-dependent acquisition pattern for
46 both 10C (p<0.001) and 230CMB (p=0.031). Membrane feeding assays on a
47 separate dataset demonstrated an association between functional transmission

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reducing activity and antibody prevalence for both 10C (p=0.017) and 230CMB (p=0.049). 17 single nucleotide polymorphisms were found in *pfs48/45* (from 126 samples), with 5 non-synonymous SNPs in the Pfs48/45 10C region.

Conclusions: We conclude there are naturally acquired antibody responses to both vaccine candidates which have functional relevance by reducing the transmissibility of infected individuals. We identified genetic polymorphisms, in *pfs48/45* which exhibited geographical specificity.

Keywords:

Plasmodium falciparum, gametocyte, gamete, transmission, immunity, polymorphism

Abbreviations:

SNP (single nucleotide polymorphism), MTBV (Malaria transmission blocking vaccines), TRA (transmission reducing activity), RDT (rapid diagnostic test), GLURP (glutamate rich protein), SMFA (standard membrane feeding assay), MSP-2 (merozoite surface protein-2), MOI (multiplicity of infection).

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68 **INTRODUCTION**

69 The recent decline in the burden of malaria, particularly in sub-Saharan Africa has
70 re-emphasized elimination as an attainable goal for many malaria endemic countries
71 [1-3]. Novel malaria control strategies that specifically aim to reduce malaria
72 transmission may be required to move from malaria control to elimination [4]. Malaria
73 transmission blocking vaccines (MTBV) are high on the priority list for malaria
74 elimination and eradication strategies [5-7]. The transmission of malaria from man to
75 mosquito depends on the presence of mature sexual stage parasites, gametocytes,
76 in the human peripheral blood. Once ingested by blood feeding mosquitoes, male
77 and female gametocytes activate to become gametes that fuse to form zygotes that
78 penetrate the mosquito midgut wall as ookinetes to form oocysts. These oocysts
79 enlarge over time to release sporozoites that migrate to the mosquito salivary glands
80 and render the mosquito infectious to human beings upon their next feeding. MTBV
81 aim to elicit antibodies that are ingested when a mosquito takes a blood meal which
82 reduce or arrest parasite development, thereby blocking transmission to the next
83 host [8]. Transmission-blocking antigens can be categorized as those that play a role
84 before zygote formation (pre-fertilization) and those that affect the subsequent
85 development of mosquito stages (post-fertilization). Pre-fertilization proteins
86 Pfs48/45 and Pfs230 are both found on the surface of gametocytes and humans
87 harbouring gametocytes in the peripheral blood are therefore exposed to these
88 proteins [8, 9]. This exposure allows the acquisition of immune response during
89 natural malaria infections. Antibody responses to both proteins have been detected
90 in naturally exposed populations and have been associated with functional
91 transmission reducing activity (TRA) [10-12]. Immune recognition may also result in
92 selective pressure that gives rise to genetic polymorphisms associated with reduced

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93 susceptibility of parasites to natural or vaccine-induced immune responses. For
94 *pfs48/45*, 5 main non synonymous genetic polymorphisms have been described
95 previously with clear geographical clustering [13]. Both the presence of naturally
96 acquired antibody responses and genetic polymorphisms in vaccine protein regions
97 are of great importance for the planning and evaluation of vaccine trials in naturally
98 exposed populations.

99 Recent work indicated that antibody responses to a Pfs230 but not a Pfs25-based
100 vaccine candidate may be recognized by naturally exposed populations [14]. Here
101 we determine naturally acquired antibody responses to MTBV candidates Pfs48/45-
102 10C and Pfs230-230CMB, explore the functionality of naturally acquired antibody
103 responses to these recombinant proteins and describe genetic polymorphisms of
104 Pfs48/45 in local isolates of *P. falciparum.*

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106 **2. MATERIALS AND METHODS**

107 *2.1 Study areas and populations*

108 Three study sites were selected to reflect different levels of transmission intensity: a
109 site of hyper endemicity in Ouahigouya, Burkina Faso, meso endemicity in
110 Bondo,Tanzania and hypo endemicity in Asutsuare in Ghana. One hundred and
111 eight children were randomly sampled from 1 school in Ghana, 200 children across
112 2 schools in Burkina Faso and 202 children across 2 schools in Tanzania using
113 sampling strategies described by Brooker *et al*. [15]. Two cross sectional surveys
114 were conducted during the peak transmission season and at the end of the dry
115 season in 2011 and 2012 at each study site [15]. Ethical permission was granted
116 from LSHTM (approval number 5946) and from local ethics committees in Burkina
117 Faso (AEP-007/05/11/CIB/CNRFP), Tanzania (Kilimanjaro Christian Medical Centre
118 IRB 2011-553) and Ghana (Noguchi Memorial Institute for Medical Research-IRB
119 040/10-11). Written consent was gained from participant's guardians prior to
120 sampling. At each survey, finger prick samples of approximately 300µL were taken in
121 BD microtainers (Becton Dickinson, Oxford, UK) for microscopy, plasma collection
122 and filter paper storage (Whatman 3MM, Maidstone, UK). Plasma was diluted to
123 1/20 in 0.05% sodium azide in phosphate buffered saline (PBS), which was stored at
124 -20℃ until use. Blood spot filter papers were air dried overnight, then sealed into
125 individual plastic bags with silica desiccant, and stored at -20℃ until use. Parasite
126 detection was done by microscopy and Rapid Diagnostic Test (RDT; Premier
127 Medical First Response, India). Clinical decision making was based on the RDT
128 result and all RDT positive individuals with reported fever in the past 24-48 hours
129 were treated for malaria according to national guidelines. Parasite counts were
130 obtained by microscopy and slides were read by two independent microscopists,

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131 both examining 100 fields. Parasite densities were determined after reading against
132 1000 leukocytes. Questionnaires were administered to participants to gain details
133 about malaria symptoms, bed net use and socio economic status.

134

135 *2.2 Antigens and Enzyme linked immunosorbent assays*

136 Pfs48/45-10C was obtained from the chimeric R0-10C vaccine protein produced at
137 the cGMP-facility of Gennova, India, using the standard proposed medium
138 composition as described by Theisen *et al.* [16]. The R0-10C vaccine protein
139 includes 10 cysteine molecules spanning epitopes I-III from the C terminal domain of
140 Pfs48/45, fused to GLURP-R0. R0 was firstly cleaved from Pfs48/45-10C and
141 successful removal of GLURP-R0 was confirmed by testing plasma samples from
142 GLURP-vaccinated volunteers [17] (see supporting information). Pfs230-230CMB
143 was obtained as a transmission blocking candidate that was developed by
144 Fraunhofer USA Center for Molecular Biotechnology. The recombinant protein
145 230CMB encompasses the pro-domain and part of the first cysteine motif domain
146 corresponding to amino acids 444-730 [18]. Additional asexual stage antigens were
147 included to contrast sexual stage antibody responses to asexual stage antibody
148 responses. These recombinant asexual proteins were; apical membrane antigen
149 (AMA-1 3D7, Biomedical Primate Research Centre, Rijswijk, the Netherlands),
150 merozoite surface protein 1_{19} (MSP-1_{19} Wellcome allele, provided by Patrick Corran,
151 London School of Hygiene & Tropical Medicine with permission of Tony Holder) and
152 R2 region of GLURP, provided by Michael Theisen, Statens Serum Institut,
153 Copenhagen.

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155

156 *2.3 Enzyme linked immunosorbent assays*

157 10C and 230CMB antibodies were quantified as follows; 96 well Maxisorp NUNC
158 plates (Nalge Nunc International Corp., Naperville, IL, USA) were coated overnight at
159 4℃ with 100µl per well of 0.1µg/ml of antigen dilu ted in PBS. Plates were blocked
160 for 30 minutes with 150µl of 5% non-fat skimmed milk (Marvel, Premier International
161 Foods Ltd., Spalding, UK) in PBS. Following this, plates were washed 3 times with
162 PBS, and 100µl of test serum was diluted to 1/500 in PBS (with 1% milk and 0.05%
163 Tween 20), and incubated on the plates for 4 hours at room temperature. Plates
164 were then washed 3 times as before and incubated with 100µl per well of human-
165 IgG-HRP (Pierce Biotechnology Inc., Rockford, IL, USA) diluted to 1/40,000 in PBS
166 with 0.05% Tween 20, for 2 hours at room temperature. Next plates were washed 4
167 times, then 100µl of tetramethylbenzidine substrate (TMB) solution was added per
168 well and incubated for 20 minutes. Reactions were stopped using 50µl per well of
169 0.2M sulphuric acid and optical densities were measured at 450nm (Bio-Rad iMark
170 Microplate Reader, Hertfordshire, UK). Serum from an expatriate with established
171 high levels of sexual stage immune responses and functional transmission reducing
172 activity (TRA) was used as a positive control [19] and titrated from 1/200 in doubling
173 dilutions 7 times to produce a standard curve. Antibody responses were quantified
174 against asexual antigens MSP-1_{19}, AMA-1 and, GLURP as described elsewhere
175 [20]. For all assays, averaged sample ODs were normalized (using the midpoint
176 dilution as reference), against a titration curve fitted to the positive control sample by
177 least squares minimisation using a three variable sigmoid model and assigning an
178 arbitrary value of 1000u/mL to calculate titre [20, 21]. The mixture model was used to
179 distinguish positive and negative samples by fitting test sample ODs to two Gaussian

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180 distributions using maximum likelihood methods in STATA (Version 11, Statacorp,
181 Texas, USA). The mean OD of the seronegative (the test samples with low ODs)
182 population plus 3 standard deviations was used as the cut off value. This resulted in
183 a cut off OD value above which a sample was considered antibody positive of; 0.17
184 for AMA-1, 0.23 for MSP-1_{19}, 0.2 for GLURP, 0.35 for 10C and 0.44 for 230CMB [10,
185 22].

186

187

188 *2.4 Analysis of functional transmission-blocking immunity*

189 While it was not possible to perform membrane feeding assays in this study where
190 small-volume finger prick blood samples were obtained, we prospectively performed
191 the 10C and 230CMB ELISA on a sample set that was longitudinally collected from
192 gametocyte carriers before and after the acquisition of gametocytes and sexual
193 stage immunity. These samples (58 samples from 25 donors) had a known
194 functional transmission-blocking phenotype in the standard membrane feeding assay
195 (SMFA) [10]. The methodology for SMFA and details of these samples are described
196 in detail elsewhere [10].

197

198 *2.5 Sequencing of the pfs48/45 gene*

199 Samples were selected at random for sequencing of the *pfs48/45* gene. In Burkina
200 Faso and Tanzania, this selection was made from microscopy-positive parasite
201 carriers. In Ghana, where microscopy data was not available at the time of
202 sequencing, we selected samples regardless of asexual parasite presence but first

203 confirmed parasite prevalence by nested PCR, which was performed according to
204 standard methods [23]. In total we obtained 126 readable sequences for samples
205 from Burkina Faso (n=39), Ghana (n=39) and Tanzania (n=48). Sequencing was
206 performed as described elsewhere [24]. Briefly, DNA was extracted from filter paper
207 blood spots using the QIAamp DNA Micro Kit (Qiagen™ Hilden, Germany) and the
208 dried blood spot protocol according to manufacturer's instructions. Since *pfs48/45* is
209 large, it was amplified and sequenced between nucleotides 22 and 1326, in 2 pieces
210 [24]. Firstly nested PCR was performed, then the secondary PCR product was
211 purified using the QIAquick PCR purification kit (Qiagen™ Hilden, Germany), and
212 sequencing PCR was performed using the BigDye V3.1 sequencing kit (Applied
213 Biosystems, San Francisco, CA, USA). Products were ethanol precipitated then
214 sequenced on an ABI3730 sequencer (Applied Biosystems, San Francisco, CA,
215 USA), and chromatograms analyzed using CLC Sequence Viewer 6 (Cambridge,
216 MA, USA). To gauge the number of clones per sample, MSP-2 genotyping of
217 asexual parasites was performed using capillary electrophoresis as described
218 elsewhere [25, 26]. GeneMapper version 4.0 (Applied Biosystems, Paisley, UK) and
219 FSTAT version 293 (Jérôme Goudet, Lausanne, Switzerland) software was used to
220 analyze results and calculate the expected heterozygosity.

221

222 *2.6 Statistical analysis*

223 All statistical analyses were performed using STATA Version 11 (Statacorp, Texas,
224 USA) and graphical presentation of data was done using GraphPadPrism Version 5
225 (GraphPad Software Inc., La Jolla, USA). Study participants were categorized into 3
226 age categories; 3-8, 9-11 and >11 to determine age dependant antibody acquisition

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227 patterns. Pearson's Chi Square and Spearman's Rank were used to determine the
228 association between categorical and continuous variables; odds ratio's with 95%
229 confidence intervals (95% CI) and correlation coefficients were used to quantify
230 effect sizes. Generalized estimating equations (GEE) were used to determine
231 associations between antibody prevalence and age, season, site and parasite status,
232 adjusting for multiple observations per individual.

233

234 **RESULTS**

235 A total of 208 samples were collected from Ghana (108 individuals), 521 from
236 Tanzania (202 individuals) and 389 from Burkina Faso (200 individuals), from
237 participants sampled during the peak transmission season and at the end of the dry
238 season for Tanzania and Ghana, and during the peak transmission season and in
239 the middle of the dry season for Burkina Faso (Table 1). The majority of children
240 were sampled in both surveys, with 5.5-7.9% of children sampled just once. Overall
241 asexual parasite prevalence by microscopy was 39.2% (148/378) in Burkina Faso,
242 12.3% (64/519) in Tanzania and 6.1% (11/180) in Ghana; gametocyte prevalence
243 was 10.1% (38/378) in Burkina Faso, 1.7% (9/520) in Tanzania and 0% (0/180) for
244 Ghana. The prevalence of both asexual parasites (OR= 1.01, 95% CI 0.94-1.09;
245 $p=0.70$) and gametocytes (OR= 1.00, 95% CI, 0.88-1.15; $p=0.94$) did not vary
246 significantly with age in this school-age population, after adjusting for season and
247 study setting. Asexual parasite density in parasite positive individuals declined with
248 increasing age ($\beta=-0.079$, se=0.022; $p<0.001$); and gametocyte density in
249 gametocyte carriers was not associated with age ($\beta=-0.013$, se=0.022; $p=0.55$), after
250 adjusting for season and study setting (Table 1). Asexual parasite prevalence was

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251 significantly higher in the transmission season compared to the dry season (OR=
252 2.00, 95% CI 1.50-2.68; p<0.001), but this seasonality was not observed for patent
253 gametocyte prevalence (OR= 1.18, 95% CI 0.65 - 2.14 p=0.59), after adjusting for
254 age and study setting.

255 The complexity of infection was determined by MSP2 genotyping for a subset of the
256 samples that were sequenced for *pfs48/45*, Ghana (n=27), Tanzania (n= 37), and
257 Burkina Faso (n=34). Multiplicity of infection (MOI) followed the same between-
258 country pattern as parasite prevalence: Ghana had the lowest MOI, with an average
259 of 1.96, followed by Tanzania with 3.35, and Burkina Faso with 3.88 (Table 2). Allelic
260 richness, a measure of genetic diversity, was lowest in Ghana (14.0), followed by
261 Tanzania (38.8) and Burkina Faso (46.9). The heterozygosity index showed a similar
262 pattern with 0.903 for Ghana, 0.970 for Tanzania and 0.973 for Burkina Faso (Table
263 2).

264

265

266

267 **Antibody responses in relation to age, parasite carriage, season and study**
268 **setting**

269 Antibody profiles for asexual stage antigens AMA-1, MSP-1_{19} and GLURP, and
270 transmission blocking vaccine candidates 10C and 230CMB are shown in table 3.
271 Combining all age categories, the proportion of people antibody positive for any
272 asexual antigen was 77.1% (300/389) for Burkina Faso, 85.0% (443/521) for
273 Tanzania and 31.1% (64/206) for Ghana (Figure 1). The overall proportion positive

274 for 10C antibodies was considerably lower with 18.0% (69/383) antibody prevalence
275 in Burkina Faso, 15.1% (66/438) in Tanzania and 1.9% (4/206) in Ghana; and
276 antibody titre followed the same pattern (Table 3). 230CMB antibody prevalence was
277 12.2% (47/384) in Burkina Faso, 18.9% (83/439) in Tanzania and 6.3% (13/205) in
278 Ghana (figure 1). The prevalence and density of antibodies against all antigens were
279 all correlated ($p<0.001$ for all comparisons; Table 4). Overall, the prevalence of
280 antibody responses to any of the asexual stage antigens increased significantly with
281 age in years (OR= 1.12, 95% CI, 1.05 -1.21; p=0.001), after adjusting for concurrent
282 parasite carriage, season and study setting. When this age-dependency of asexual
283 antibody prevalence trend was examined per study setting, there was a significant
284 positive association in Burkina Faso (p=0.022) and Ghana (p=0.012) but not
285 Tanzania (p=0.41). Antibody prevalence to 10C increased with age (Figure 1), but
286 this trend was only statistically significant in Burkina Faso (OR= 1.39, 95% CI 1.18-
287 1.65; $p<0.001$). Similarly, antibody responses to 230CMB increased with age, but
288 only in Burkina Faso (OR= 1.21, 95% CI 1.02 -1.44; p=0.031) and Tanzania (OR=
289 1.22, 95% CI, 1.07 -1.38, p=0.003), with no clear pattern for Ghana (p=0.32). The
290 concurrent presence of asexual parasites was significantly associated with the
291 prevalence of antibodies against any asexual stage antigen (OR 3.55, 95% CI 2.32-
292 5.42, $p<0.001$) and against the individual asexual antigens; AMA-1, MSP-1_{19} and
293 GLURP ($p\leq0.012$) and antibody titre for AMA-1 ($p<0.001$), GLURP ($p<0.001$) but not
294 MSP-1_{19} (p=0.64). The concurrent presence of asexual parasites was also
295 associated with a higher prevalence of antibodies against 10C (OR 2.01, 95% CI
296 1.30-3.11, p=0.002) and 230CMB (OR 1.65, 95% CI 1.06-2.57; p=0.027) and a
297 higher antibody titre for 10C (log_{10} titre: $\beta=0.35$, se=0.081, $p<0.001$) and 230CMB
298 (log_{10} titre: $\beta=0.37$, se=0.076, $p<0.001$), after adjustment for age, season and study

299 setting. There was no association between microscopically detectable gametocytes
300 and antibody prevalence for 10C (p=0.456) or 230CMB (p=0.281).

301

302 **Functional transmission reducing activity**

303 A total of 58 samples with SMFA results from a previous study were successfully
304 tested for 10C and 230CMB antibodies, of which 19/58 reduced transmission by
305 ≥50% and 9/58 reduced transmission by ≥90%. Both 10C and 230CMB antibody
306 prevalence was significantly associated with TRA at both the 50% and 90% levels
307 (Table 5).

308

309 ***pfs48/45* sequencing**

310 We sequenced *pfs48/45* in a subset of samples from Tanzania (n=48), Ghana
311 (n=39) and Burkina Faso (n=39). All variation discussed here is compared to the 3D7
312 reference sequence [27]. 17 single nucleotide polymorphisms (SNPs) were found,
313 10 of which were synonymous and 7 non-synonymous. 12 of the SNPs (5 of the non-
314 synonymous SNPs) are within the vaccine candidate region (supplementary table 1).
315 Two new non synonymous substitutions were found at nucleotide locations 396
316 (amino acid position 132) and 468 (amino acid position 156), that appeared once in
317 both Ghana and Tanzania, and are outside of the 10C recombinant protein region.
318 For Ghana, the majority of samples (69.2%) were identical to 3D7 for *pfs48/45*; for
319 Tanzania and Burkina Faso this proportion was 50.0% and 17.9%, respectively
320 (Table 2). Ghana had the lowest number of haplotypes (n=11), (defined as a specific
321 collective of SNPs), Burkina Faso had 15 and Tanzania, 21, resulting in 44 unique

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322 haplotypes overall. Ghana also had the lowest number of infections with mixed

323 *pfs48/45* haplotypes (15.4%), followed by Tanzania (43.8%) and Burkina Faso

324 (56.4%). Five SNPs were found most frequently in terms of appearing in different

325 haplotypes and also appearing in the largest numbers of samples. These are located

326 at nucleotide positions 757, 762, 911, 940 and 965 and are all non synonymous

327 amino acid changes (Table 6).

328

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330 **DISCUSSION**

331 The present study is the first to determine natural recognition of MTBV candidates
332 Pfs48/45-10C and Pfs230-230CMB in three malaria endemic settings and extends
333 our appreciation of genetic polymorphisms in *pfs48/45*. These observations need to
334 be taken into consideration in the planning of vaccine trials that have to allow for the
335 presence of protein recognition prior to vaccination and potential immune boosting
336 following natural antigen exposure.

337 Pre-fertilization and post-fertilization antigens for MBTV differ not only in their
338 mechanisms of action but also in their potential for immune-boosting following
339 natural infections. Whilst the possible immune boosting of Pfs230 and Pfs48/45
340 antibodies has been commonly mentioned [28], it is only with the availability of
341 recombinant proteins that the extent of natural immune recognition can be fully
342 explored. The availability of recombinant proteins also allows addressing immuno-
343 epidemiological questions about the rate of antibody acquisition and its relationship
344 with age and transmission intensity [29]. All studies up to date have relied on assays
345 using natural antigen from cultured gametocyte extract, limiting throughput and
346 sensitivity because of high background reactivity [10-12, 30-34]. We examined
347 antibody responses to *P. falciparum* transmission blocking vaccine candidates and
348 asexual stage vaccine candidates in three endemic settings in Africa. Multiplicity of
349 infection (MOI), allelic richness, asexual parasite prevalence and serological markers
350 of malaria exposure were used to characterize sites in Ghana, Tanzania and Burkina
351 Faso [26, 35-37]. All indices used, indicate low transmission intensity in our site in
352 Ghana and intense transmission intensity in our site in Burkina Faso. Tanzania was
353 selected as a site of meso endemicity. A reduction in malaria burden of 65.4% was
354 reported between 2003-2008, which was attributed to declining numbers of

355 mosquitoes [38]. Our results of high antibody prevalence and high parasite
356 prevalence in the youngest age group, indicate that malaria is still highly prevalent in
357 this setting [38, 39].

358 Naturally acquired antibody responses recognizing transmission blocking vaccine
359 candidates 10C and 230CMB broadly followed the pattern of asexual stage antibody
360 responses with lowest antibody responses in Ghana, the area of low transmission
361 intensity. 10C and 230CMB antibody responses were also associated with
362 concurrent asexual parasite prevalence, indicative of higher malaria exposure [40,
363 41], and a general trend of higher antibody prevalence in older children. Within our
364 sample set we found no association between gametocyte carriage, detected by
365 microscopy, and the concurrent presence of sexual stage antibodies. This is
366 unsurprising since gametocyte carriage by microscopy is commonly of short duration
367 during infections [42] and there is a lag period following gametocyte exposure before
368 antibodies are produced and can be detected [10]. Antibody prevalence thereby
369 reflects previous and not necessarily current gametocyte exposure and the
370 association between antibody responses and antigen exposure can only be
371 addressed in longitudinal studies, where gametocyte carriage is repeatedly assessed
372 by quantitative (molecular) methods. The associations of sexual stage antibody
373 responses with asexual parasite prevalence, that has repeatedly been identified as
374 an indicator of both current and previous malaria exposure [41], suggests that
375 individuals with current asexual parasite prevalence may have previously been
376 exposed to a higher number of infections with concurrent gametocytes and therefore
377 sexual stage antigen exposure.

378 The age-dependency of sexual stage malaria immunity has not been firmly
379 established [10, 12, 30, 34] and there is insufficient evidence on the longevity of

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380 antibody responses [10]. More detailed longitudinal studies across a larger age-
381 range are needed, our findings indicate that the recombinant vaccine proteins are
382 suitable tools for such studies. Importantly, we observed a highly significant
383 association between naturally acquired antibody responses recognizing 10C and
384 230CMB and functional TRA, as was previously reported for the native proteins
385 Pfs48/45 and Pfs230 [10-12, 34, 43-46]. While TRA in the supportive dataset was
386 significantly associated with antibodies against 10C and 230CMB, there may be
387 antibodies to other candidate transmission-blocking antigens that we have not ruled
388 out as contributors. In general, our assays fall short of providing definitive evidence
389 for the transmission-blocking activity of naturally acquired 230CMB and 10C
390 antibodies. Whilst, antibodies generated by immunization of rodents with 230CMB or
391 10C and monoclonal antibodies against Pfs230 and Pfs48/45 are causally
392 associated with transmission blocking activity [16, 18, 47, 48], our epidemiological
393 findings do not provide the same level of evidence. To formally conclude that
394 naturally acquired 10C and 230CMB antibodies in humans are responsible for the
395 observed transmission-blocking activity, future studies may affinity purify antibodies
396 against these two antigens and test these purified antibodies using SMFA.

397 Taken together, our findings of natural recognition of 10C and 230CMB antibody
398 responses and their association with commonly used proxy-markers of malaria
399 exposure (age, concurrent parasite prevalence, serological markers of cumulative
400 exposure to asexual stage antigens) suggest that natural parasite exposure may
401 boost vaccine induced immunity, [16, 18] and that field trials with these vaccine
402 candidates have to take into account naturally acquired immune responses to the
403 vaccine proteins.

404 For the 10C vaccine candidate, we provided relevant information on genetic variation
405 in the *pfs48/45* gene in our three field sites. We identified 5 previously documented
406 non-synonymous substitutions [13] and discovered 2 new non-synonymous
407 substitutions that were both detected once and are present outside the part of the
408 *pfs48/45* gene that forms the basis of the 10C vaccine. The genetic sequence of 10C
409 in the R0-10C vaccine was based on cultured isolate 3D7/NF54. In a recent study,
410 R0-10C induced immunity showed significant TRA (>90%) against cultured isolates
411 3D7/NF54, and NF135 which have polymorphisms in amino acids 254, 304 and 322
412 [16], indicating that these polymorphisms do not necessarily translate to lower
413 vaccine efficacy. While a substantial proportion of field isolates were different from
414 3D7/NF54 (30.8% in Ghana, 50.0% in Tanzania and 82% in Burkina Faso), it is
415 currently unknown whether this has consequences for 10C vaccine efficacy.
416 Polymorphisms on positions 757 and 940 may be of particular interest in this
417 respect, since these were commonly detected in field isolates but not in NF135, a
418 cultured strain for which efficient transmission blocking activity of anti-10C antibodies
419 was confirmed [16]. Previous research highlighted that the 5 non synonymous
420 substitutions resulted in 9 haplotypes that showed geographical clustering across
421 Africa, Asia and South America, [13, 24, 49]. These findings are in line with our study
422 which found these substitutions to be common, but resulted in a larger number of
423 haplotypes, with many being mixed infections. While we only analysed samples from
424 African settings, we also found clear geographical clustering of haplotypes. A high
425 number of mixed clone infections were seen in our samples. As it was not possible to
426 determine which SNPs belonged to different clones, mixed sequencing results were
427 designated new haplotype identities. It is possible that dominant clones may shroud
428 lower density clones resulting in an underestimation in the MOI and *pfs48/45*

429 sequencing data [37]. Genetic polymorphisms in vaccine candidates can threaten
430 success by enabling immune escape mutants [50]. The majority of non synonymous
431 substitutions found in these samples, and in previous studies are within the vaccine
432 candidate region. It is currently unclear, but of utmost importance, to determine if
433 vaccine induced immunity is likely to be efficacious against strains with a different
434 genetic composition on the functional epitopes.

435

436 In summary, we have found that antibodies against two new recombinant vaccine
437 candidates are naturally acquired, found in serum from 3 endemic regions across
438 both East and West Africa, and show age dependency in Burkina Faso. The
439 functional importance of these antibodies has been confirmed using samples with
440 SMFA data which showed a significant correlation between antibody prevalence and
441 TRA. Our samples show genetic variation within the vaccine candidate that exhibit
442 geographical clustering, the relevance of which is currently not known.

443

444

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455

456 **Conflict of interest**

457 We declare there are no competing interests.

458

459

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588 **Figure legends**

589 **Figure 1. Antibody prevalence for asexual antigens, and sexual stage antigens**
590 **10C and 230CMB, in relation to participant age group and country of origin.**
591 Individuals counted as asexual positive have antibodies to any or multiple of the
592 following antigens; AMA-1, MSP-1_{19} or GLURP. The bars show the antibody
593 prevalence in the age groups, shaded by country; white for Ghana, light grey for
594 Tanzania and dark grey, Burkina Faso. The error bars indicate the 95% confidence
595 interval and the asterisks indicates a statistically significant positive increasing trend
596 at the following levels: 0.05 – 0.01 (*), 0.01- 0.001 (**), and <0.001 (***).
597

598 **Table legends**

599 **Table 1**. **Characteristics of survey participants** IQR, interquartile range based on
600 25th and 75th percentiles, GM, geometric mean, CI, 95% confidence interval. *
601 derived from the dataset as a whole. This table contains data for participants where
602 we know their age (38 people are missing from this table).
603

604 **Table 2. Multiplicity of infection (MOI) and *pfs48/45* sequencing results by**
605 **study location.** *A sub population of the sequenced samples were assessed to
606 determine multiplicity of infection.
607

608 **Table 3. Antibody prevalence and density for asexual stage and sexual stage**
609 **antigens**. Titres refer to antibody positive individuals only. GM, geometric mean, CI,
610 95% confidence interval.
611

612 **Table 4. Associations between antibody titres for the different antigens**. The
613 spearman correlation coefficient is presented with the related p-value.

614

615

616 **Table 5. Relationship between sexual stage antibody responses and functional**
617 **transmission reducing activity (TRA).** Transmission reducing activity was
618 categorized at $\geq$50% and $\geq$90%. Odds ratios are presented with 95% confidence
619 intervals, and the associated p-values.

620

621 **Table 6. Single nucleotide polymorphisms (SNPs) in *pfs48/45* per study**
622 **location.**

623

624 **Supplementary information**

625

626 **Figure S1 (a) Sodium dodecylsulphate (SDS) polyacrylamide gel**
627 **electrophoresis and, (b) Western blotting analysis of cut and uncut protein**.
628 Lanes 1 and 4 contain molecular weight markers (SeeBlue Pre-stained standard
629 (Invitrogen LC5625) lanes 2 and 5 have 100% uncut protein and the cleaved product
630 is in lanes 3 and 6.

631

632 **Figure S2**. **The relationship between the density of 10C and 230CMB antibody**
633 **responses and transmission reducing activity (reduction in oocyst density).**
634 Open circles represent 10C antibody responses and black triangles represent
635 230CMB antibody responses.

636

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1 **Table 1**. **Characteristics of survey participants** IQR, interquartile range based on 25th and 75th percentiles, GM, geometric mean,

2 CI, 95% confidence interval. * derived from the dataset as a whole. This table contains data for participants where we know their

3 age (38 people are missing from this table).

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	Ghana			Tanzania			Burkina Faso		
Age group	≤8.0	9-11	>11	≤8.0	9-11	>11	≤8.0	9-11	>11
Number	55	58	67	40	175	305	195	163	22
Age, median (IQR)	7 (6-8)	10 (9-10)	12 (13-14)	8 (7-8)	10 (10-11)	14 (12-15)	7 (6-8)	10 (9-11)	12 (12-13)
Gender, % female (n/N)	45.5 (25/55)	37.9 (22/58)	34.9 (22/63)	75.7 (28/37)	58.9 (103/175)	47.5 (145/305)	47.2 (92/195)	53.7 (87/162)	33.3 (7/21)
Asexual prevalence, % (n/N)	3.6 (2/55)	10.3 (6/58)	4.5 (3/66)	22.5 (9/40)	11.4 (20/175)	11.5 (35/304)	38.0 (74/195)	40.7 (66/162)	38.1 (8/21)
Asexual density, GM (IQR)	119 (32-440)	321 (128-480)	142 (64-400)	1204 (1120-2707)	501 (128-1960)	384 (112-996)	970 (245-2796)	462 (120-1196)	818 (397-2304)
Gametocyte prevalence, % (n/N)	0 (0/55)	0 (0/58)	0 (0/67)	2.5 (1/40)	2.3 (4/175)	1.3 (4/305)	9.7 (19/195)	10.5 (17/162)	9.5 (2/21)
Gametocyte density, GM (IQR)	n/a	n/a	n/a	8.0 (8.0-8.0)	16.0 (8-40)	33.8 (24-56)	18.3 (8-38)	15.9 (8-20)	11.0 (8-15)

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8 **Table 2. Multiplicity of infection (MOI) and *pfs48/45* sequencing results by study location.** *A sub population of the
9 sequenced samples were assessed to determine multiplicity of infection.

	Ghana	Tanzania	Burkina Faso
Number of analyzed samples (MOI)	27*	37*	34*
Average number of clones	1.96	3.35	3.88
% single clone infections	44.0	10.8	17.6
Allelic richness	14.0	38.8	46.9
Expected heterozygosity	0.903	0.970	0.973
Number of analyzed samples (sequencing)	39	48	39
***pfs48/45* haplotypes, number**	11	21	15
***pfs48/45* haplotypes,% 3D7**	69.2	50.0	17.9
***pfs48/45* haplotypes, % mixed clone**	15.4	43.8	56.4

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Table 3. Antibody prevalence and density for asexual stage and sexual stage antigens. Titres refer to antibody positive individuals only. GM, geometric mean, CI, 95% confidence interval.

	Ghana			Tanzania			Burkina Faso		
Age group	≤8.0	9-11	>11	≤8.0	9-11	>11	≤8.0	9-11	>11
AMA-1, prevalence (n/N)	14.3 (7/49)	24.6 (14/57)	44.3 (27/61)	92.1 (35/38)	79.8 (126/158)	79.9 (226/283)	61.5 (118/192)	67.7 (107/158)	90.9 (20/22)
Titre, GM (95% CI)	854 (290-2520)	700 (395-1240)	517 (346-773)	1194 (822-1738)	827 (678-1008)	1080 (928-1259)	909 (728-1136)	1129 (896-1422)	2364 (1198-4667)
MSP-1, prevalence (n/N)	18.9 (10/53)	0 (0/58)	19.7 (13/66)	32.5 (13/40)	40.8 (71/174)	44.6 (136/305)	24.2 (47/194)	26.5 (43/162)	15 (3/20)
Titre, GM (95% CI)	594 (389-905)	0 (0-0)	1240 (821-1874)	1106 (656-1867)	1300 (1029-1643)	1085 (926-1271)	1179 (844-1646)	931 (648-1338)	548 (187-1610)
GLURP, prevalence (n/N)	7.6 (4/53)	12.3 (7/57)	24.2 (16/66)	77.5 (31/40)	60.1 (104/173)	66.8 (197/295)	32.4 (56/173)	61.9 (91/147)	63.2 (12/19)

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Titre, GM (95% CI)	1116 (156-7990)	841 (489-1446)	539 (382-761)	871 (605-1255)	879 (739-1045)	1195 (1028-1389)	968 (728-1288)	1433 (1126-1824)	1317 (528-3285)
10C, prevalence (n/N)	1.82 (1/55)	0 (0/57)	1.5 (1/66)	18.8 (6/32)	11.8 (17/144)	16.5 (43/261)	8.33 (16/192)	28.1 (45/160)	31.8 (7/22)
Titre, GM (95% CI)	50	0	53	77 (45-131)	107 (75-151)	86 (71-104)	81 (60-110)	80 (70-91)	70 (49-99)
230CMB, prevalence (n/N)	5.5 (3/55)	1.8 (1/57)	12.1 (8/66)	12.5 (4/32)	13.2 (19/144)	22.9 (60/262)	6.8 (13/192)	16.8 (27/161)	22.7 (5/22)
Titre, GM (CI)	623 (192-2021)	202	284 (190-426)	273 (78-957)	299 (202-442)	307 (256-367)	384 (204-722)	265 (235-299)	598 (139-2574)

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22 **Table 4. Associations between antibody titres for the different antigens**. The spearman correlation coefficient is presented
23 with the related p-value.

antibody	AMA-1	MSP-1	GLURP	10C
AMA-1				
MSP-1	r=0.39, p<0.001			
GLURP	r=0.55, p<0.001	r=0.64, p<0.001		
10C	r=0.38, p<0.001	r=0.30, p<0.001	r=0.44, p<0.001	
230CMB	r=0.32, p<0.001	r=0.33, p<0.001	r=0.42, p<0.001	r=0.53, p<0.001

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30 **Table 5. Relationship between sexual stage antibody responses and functional transmission reducing activity (TRA).**

31 Transmission reducing activity was categorized at ≥50% and ≥90%. Odds ratios are presented with 95% confidence intervals, and

32 the associated p-values.

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Antibody prevalence	TRA ≥50%		TRA ≥90%	
	OR (95% CI)	p-value	OR (95% CI)	p-value
10C	5.54 (1.21-25.43)	0.028	7.04(1.41-35.13)	0.017
230CMB	8.48(1.91-37.64)	0.005	4.69(1.00-21.85)	0.049

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37 **Table 6. Single nucleotide polymorphisms (SNPs) in *pfs48/45* per study location.**

location and type of nucleotide change			SNP frequency by country			
Pfs48/45 *epitope location*	**nucleotide location (corresponding amino acid if changed)**	**amino acid change**	**Ghana**	**Tanzania**	**Burkina Faso**	**Total**
epitope V	328		1	0	0	1
	336		0	0	1	1
	396 (132)	I=T	1	0	0	1
	419		0	1	0	1
	468 (156)	F=V	0	1	0	1
epitope II & III	492		1	0	0	1
	502		0	1	0	1
	556		1	1	0	2
	753		1	0	0	1
	757 (253)	K=E	2	13	5	20
	762 (254)	N=K	9	21	12	42

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epitope I	911 (304)	V=D	7	8	3	18
	940 (314)	L=I	2	6	29	37
	965 (322)	S=N	7	6	4	17
	989		1	0	0	1
	1023		2	1	0	3
	1047		0	1	0	1

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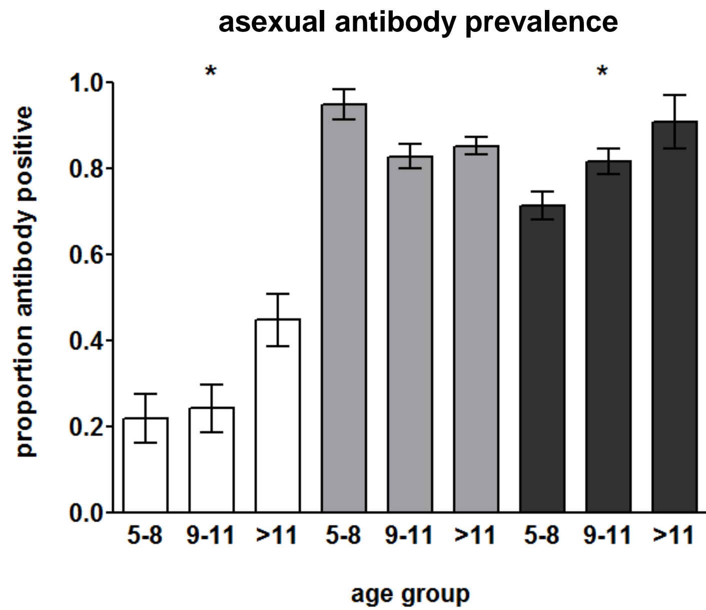

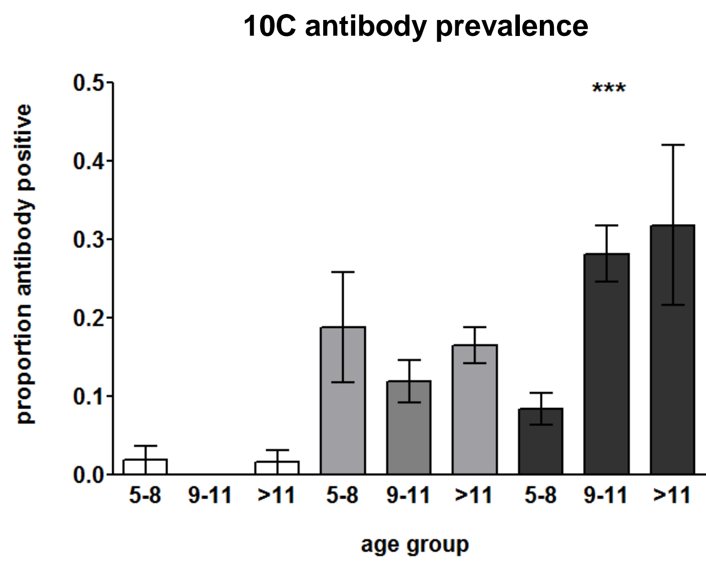

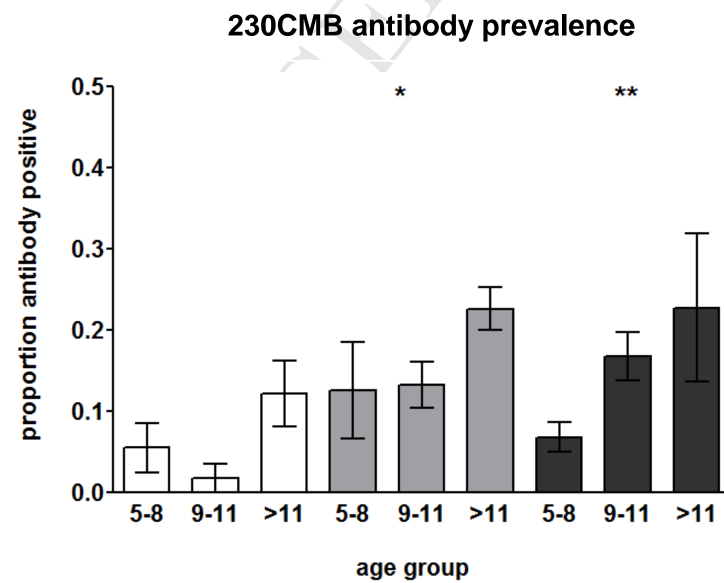

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FIG. 1 Antibody prevalence for asexual antigens, and sexual stage antigens 10C and 230CMB, in relation to age group and country of origin. Individuals counted as asexual positive have antibodies to any or multiple of the following antigens; AMA-1, MSP-1_{19} or GLURP. The bars show the antibody prevalence in the age groups, shaded by country; white for Ghana, light grey for Tanzania and dark grey, Burkina Faso. The error bars indicate the 95% confidence interval, and the asterisks indicates a statistically significant positive increasing trend at the following levels: 0.05 – 0.01 (*), 0.01- 0.001 (**), and <0.001 (***).

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1 **Supplementary methods and results**

2 A factor Xa site was included between R0 and 10C to enable cleavage and allow
3 quantification of sexual stage antibodies. To cleave R0, the following mastermix was
4 prepared; 200 µl of 100% properly folded R0-10C (300µg) was incubated with 20µl of
5 100mM of $CaCl_2$, 20µl of 1% DOC in Tris-buffer and 4µl of undiluted Factor Xa
6 Protease (New England BioLabs Inc, Leiden, The Netherlands) overnight at room
7 temperature.

8 To confirm complete cleavage, product was visualized using sodium dodecyl
9 sulphate (SDS) polyacrylamide gel electrophoresis and a 4-12% NuPAGE (R) gel
10 (Invitrogen Life Technologies, Leek, The Netherlands). 15µl of cleaved product was
11 incubated with 15µl of Tris-Glycine SDS, for 20 minutes at 80℃, then 15µl of product
12 was visualized on the gel (figure S1).

13 Protein reactivity against epitope 45.2b of Pfs48/45, was confirmed using Western
14 Blotting. Protein bands were transferred from a second SDS gel to Hybond-C-extra
15 paper (Amersham), which was blocked with 5% milk in PBS for 30 minutes on a
16 shaking incubator. The gel was incubated in 1.5µg/ml of HRP-mAb85RF45.2b
17 (epitope 45.2b of Pfs48/45) in PBS with 0.05% Tween-20 for 2 hours on a shaking
18 incubator. The gel was then incubated with peroxidise substrate Kit Vector SG
19 (SK4700, Vector Laboratories), which enabled identification of protein bands that
20 reacted against epitope 45.2b.

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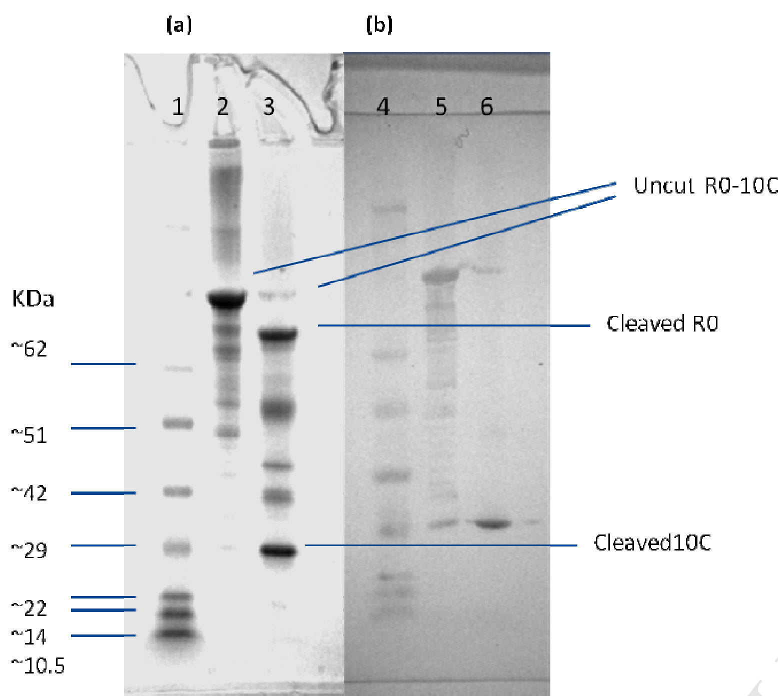

23 **Figure S1 (a) Sodium dodecylsulphate (SDS) polyacrylamide gel**

24 **electrophoresis and, (b) Western blotting analysis of cut and uncut protein**.

25 Lanes 1 and 4 contain molecular weight markers (SeeBlue Pre-stained standard

26 (Invitrogen LC5625) lanes 2 and 5 have 100% uncut protein and the cleaved product

27 is in lanes 3 and 6.

28

29 Figure S1 shows the product of the cleavage reaction (a), and the reactivity of the

30 products to epitope 2b (b) in a Western blot. Uncut R0-10C is 89.8 KDa in size, which

31 following cleavage divides into R0, 58.3 KDa in size, and 10C-6His which is 31.5

32 KDa. Figure S1 (b) shows there is reactivity against epitope 2b Pfs48/45 to two

33 bands in the cleaved product, which are at the correct size for cleaved 10C and a

34 minor band for uncleaved protein, which confirms that there is some uncut R0-10C in

35 the ‘cleaved’ product.

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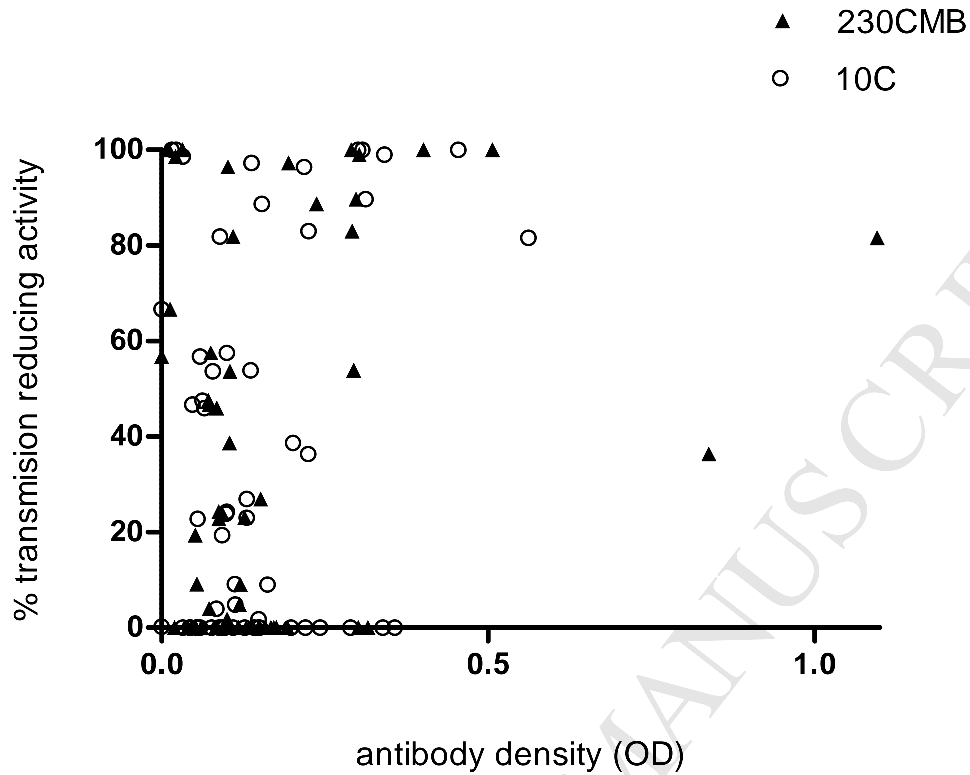
36 Reactivity of 10C was checked with a two site ELISA using anti-Pfs48.45 mAbs
37 recognizing epitopes 1 and 2B, which was otherwise performed as before. Samples
38 were assayed from known gametocyte carriers who had antibodies against Pfs48/45
39 and had been shown to demonstrate transmission reducing activity using membrane
40 feeding assays. To confirm specificity, a control sample was used from an individual
41 who had been vaccinated with GLURP. High reactivity against gametocyte positive
42 individuals and low reactivity against the GLURP vaccinated individual indicated the
43 cleaved product was just 10C.

44 10C was then purified by gel filtration on a Sephacryl S-200 column (GE Healthcare,
45 The Netherlands) size exclusion column to remove the uncut protein. 150µl of 10C
46 was mixed with 1000µl of buffer (EDTA 250mM, Tris 1M pH 8.8, NaCl 100mM, 0.8%
47 sodium deoxychelate in ddH_20), then loaded onto the column and run for 3 hours to
48 enable separation. Fractions corresponding to 10C were pooled and concentrated
49 according to their expected molecular weight using VivaSpin columns (GE
50 Healthcare, The Netherlands) which were centrifuged at 4000 rpm for 25 minutes.
51 Following this, supernatant was checked on SDS and Western blot once more,
52 concentration estimated using Nanodrop and BCA and reactivity confirmed once
53 more using ELISA. Product was concluded to be free of R0.

54

55 Figure S2 demonstrates the relationship between the density of 10C and 230CMB
56 antibody responses and transmission reducing activity (reduction in oocyst density),
57 which is plotted as a continuous variable. This data must be interpreted with caution,
58 since it has been repeatedly demonstrated only higher levels of transmission
59 reducing activity are reproducible (Van der Kolk, 2004).

60



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62 **Figure S2**. **The relationship between the density of 10C and 230CMB antibody**

63 **responses and transmission reducing activity (reduction in oocyst density).**

64 Open circles represent 10C antibody responses and black triangles represent

65 230CMB antibody responses.

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