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# Unravelling the immune signature of Plasmodium falciparum transmission reducing immunity

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8 **immunity**

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Plasmodium gametocytes develop from their asexual progenitors and are the only malaria parasite life-stage infective to mosquitoes. In preparation for their development in the mosquito, gametocytes cease to express many proteins involved in the parasites cycle of asexual replication, and upregulate others that are involved in sexual development [1,2]. Some of these have essential roles in mosquito-stage development [3,4], which culminates in the insect becoming infectious to humans.

In surveys where mosquitoes were fed directly on the skin or on a blood sample, it was noted that some gametocytaemic individuals including those with high gametocyte densities were not infectious [5,6]. This may be associated with naturally acquired antibodies that interfere with parasite development within mosquitoes to reduce or prevent mosquito infection [7,8]. The role of antibodies in determining transmission efficiency can be tested in mosquito-feeding assays, in which purified antibodies are added to a mosquito blood meal that contains gametocytes [9,10]. Evidence for naturally acquired transmission-reducing activity (TRA) is provided if test antibodies in endemic serum cause a reduction in the number of developing Plasmodium oocysts relative to mosquitoes fed the same infectious blood meal without test antibodies. TRA can be experimentally induced after immunisation of animals with inactivated Plasmodium gametocytes or gametes [8,11]. The gametocyte and gamete proteins P48/45 and P230 have been identified as targets of transmission-blocking monoclonal antibodies (mAb), which act by inhibiting the protein's role in gamete fertilisation in the mosquito gut [3,4,12]. Monoclonal antibodies specific to the zygote and ookinete proteins P25 and P28 were also shown to block transmission, by inhibiting ookinete invasion of the midgut epithelium13. All four have been produced as recombinant proteins that can induce antibody-mediated TRA in animal models [13–15].

Because P25 and P28 mRNA is translationally repressed until zygote formation [16], antibodies specific to these proteins appear absent in endemic populations [17,18]. In contrast, responses to P48/ 45 and P230 are commonly observed in naturally infected individuals [10,19,20]. The presence of antibodies to Plasmodium falciparum Pfs48/45 and Pfs230 in endemic sera has been associated with TRA in several, but not all sero-epidemiological surveys [10,21–28]. Importantly, many individuals with functional TRA do not have measurable antibodies against these two proteins [10,23,25,28–31]. Despite these observations, there has been no attempt to demonstrate the contribution of naturally acquired α-Pfs48/45 and α-Pfs230 antibodies to transmission inhibition, and little investigation of alternative targets of natural TRA. The gametocyte proteome has now been described in detail [32,33]. Utilising the protein microarray platform, genome scale data sets have been combined with functional and immunological data to provide valuable insight into mechanisms and markers of malaria humoral immunity [34,35].

93  Here, we aim to investigate the immune signature of naturally occurring antibody-mediated
94  TRA, to expand and prioritise antigenic targets for functional characterisation as biomarkers,
95  or transmission-blocking vaccine candidates. To this purpose, we utilise a protein microarray
96  comprising an inclusive selection of proteins expressed during gametocyte development. We
97  assess antibody responses to these proteins and to correctly folded Pfs48/4536 and
98  Pfs23015 in 648 malaria-exposed individuals from Burkina Faso, the Gambia, Cameroon,
99  and from migrants from the Netherlands. Using purified total IgG, we assess the functional
100  TRA of antibodies from the same individuals by determining their effect on mosquito
101  infection density in standard membrane feeding assays (SMFA) with cultured *P. falciparum*
102  gametocytes and *Anopheles stephensi* mosquitoes. Our analysis reveals significant
103  associations between high-level TRA and responses to Pfs48/45, Pfs230 and 43 novel
104  gametocyte proteins. For a subset of 366 gametocyte-positive donors who had provided
105  blood samples to *Anopheles gambiae* s.s. or *An. coluzzii* mosquitoes in field-based
106  membrane-feeding assays, we determine the association of these antibody responses with
107  mosquito infection rates during natural infections. For Pfs48/45 and Pfs230, we provide
108  functional data that demonstrate their role in natural TRA. For one of the newly identified
109  proteins (PfGEST), we provide experimental data that does not support its functional role in
110  natural TRA.

111

112  **Results**

113  **Antibody responses to gametocyte antigens**. Plasma was collected in epidemiological
114  studies in Burkina Faso [37–41], Cameroon [42,43] and the Gambia [24,44–48], as well as
115  from Dutch migrants who had lived for several years in malaria-endemic areas and reported
116  repeated malaria episodes (Table 1). Individuals from malaria-endemic areas were all
117  asymptomatic when sampled, and were either recruited randomly from the community (n=
118  42), or based on the observation of malaria parasites (n = 273) or specifically gametocytes
119  (n = 276) in blood smears. For a selection of individuals from malaria-endemic areas (n =
120  498), infectivity to mosquitoes at the moment of sample collection was determined by
121  offering a venous blood sample to *Anopheles gambiae* s.s. or *An. coluzzii* in a direct
122  membrane-feeding assay (DMFA) (Table 1). Dutch migrants had no DMFA performed and
123  had either recently returned from extended periods in endemic areas (within 6 months after
124  return; n = 8) or had returned more than a year before sampling (n = 49). Those who had
125  returned more than a year before sampling had lived in areas with endemic *P. falciparum*
126  transmission for longer than 15 years, and reported between 2 and >80 clinical malaria
127  episodes during that time.

128  Antibody responses to Pfs48/45 (Pfs48/45-10C [36]) and Pfs230 (230CMB [15]) were
129  determined by enzyme-linked immunosorbent assay (ELISA) using recombinant, correctly
130  folded proteins. A protein microarray was constructed to assess responses to other
131  gametocyte antigens. This array included a total of 315 proteins (Supplementary Data 1) that
132  were broken up into 528 overlapping fragments <1000 amino acids in length (overlaps of 17
133  amino acids) for protein expression in an *Escherichia coli* based in vitro transcription–
134  translation (IVTT) system [49,50]. Antigens on the array included proteins previously
135  implicated as eliciting TRA after immunisation, proteins involved in early gametocyte
136  development as possible markers of gametocyte exposure1, markers of asexual parasite
137  exposure [51] and proteins expressed in gametocytes based on recent proteomic analysis
138  [52]. To ensure that the array included most potential antibody targets involved in TRA, we
139  preferentially included proteins that had transmembrane domains, signal peptides or GPI
140  anchors. Specificity of proteins to gametocytes was not a prerequisite for inclusion on the
141  array, however 228/315 proteins had consensus evidence for enrichment in gametocytes
142  based on a recent integration analysis of 11 available proteomics data sets [53]
143  (Supplementary Data 1). Among donors from malaria-endemic regions, the prevalence and
144  magnitude of antibody responses to Pfs48/45 and Pfs230 increased with age (Fig. 1a–d). In
145  contrast, the overall breadth (linear regression (LinR): p < 0.001) and magnitude (analysis of
146  variance (ANOVA): p = 0.013) of response to antigens on the microarray decreased with age
147  (Fig. 1e, f). The same associations were observed when analyses were restricted to donors
148  from Burkina Faso (n = 225), the site with the widest age range of donors (Supplementary
149  Fig. 1).

150  **Antibody responses and transmission-reducing activity (TRA)**. To assess functional
151  TRA, IgG was purified from the sera of all 648 individuals and provided to *Anopheles*
152  *stephensi* mosquitoes together with a blood meal containing cultured NF54 or transgenic
153  NF54HT-GFP-Luc gametocytes in standard membrane feeding assays (SMFA) [9,54]. If the
154  donor IgG caused ⩾80% reduction in oocyst formation relative to control mosquitoes (TRA
155  ⩾80%), the SMFA was repeated with separate gametocyte culture material. In total, 3.3%
156  (22/648) of the antibody samples caused replicable TRA of ⩾ 90% (Table 1). On a
157  continuous scale, TRA decreased with age, but was not significantly associated with
158  gametocyte density at the point of sampling (LinR: p = 0.269) (Supplementary Fig. 2).
159  Because previous gametocyte exposure is associated with TRA28, comparisons between
160  sub-groups were nevertheless adjusted for gametocyte density. Though intermediate TRA
161  may be relevant for transmission there are uncertainties about its reliable measurement in
162  the SMFA [55,56]. TRA was therefore dichotomised into ⩾90% (high-level TRA) and <10%
163  TRA (no TRA) before assessing associations with antibody responses. The group without

164  evidence for TRA was refined by excluding individuals without microscopically detectable
165  gametocyte densities at the time of sampling, resulting in 22 individuals with high-level TRA
166  activity and 254 individuals who were exposed to gametocytes (and thus likely to respond to
167  gametocyte antigens) but showed no evidence for TRA. Using these two categories, high-
168  level TRA was significantly more likely in individuals seropositive for α-Pfs230 (odds-ratio
169  (OR) from logistic regression (LogR) 7.9 (95% confidence interval (CI): 2.8–22.8), $p < 0.001$),
170  α-Pfs48/45 (OR 4.4 (95% CI: 1.5–12.9), $p = 0.007$) or either antigen (OR 5.90 (95% CI: 2.1–
171  16.7), $p = 0.001$) (Supplementary Table 1, Supplementary Data 2). Antibody density was
172  also higher for Pfs230 (LinR: $p < 0.001$) and Pfs48/45 ($p < 0.001$) in individuals exhibiting
173  high-level TRA (Fig. 2a, b; Supplementary Data 2).

174  In addition to Pfs230 and Pfs48/45, the average magnitude of response to each microarray
175  target was also higher in individuals with high-level TRA (students t-test: $p = 0.012$) (Fig. 2d),
176  whilst the breadth of response by each individual was not statistically significantly different
177  between individuals with high-level TRA compared to those with no evidence of TRA (LinR:
178  $p = 0.89$) (Fig. 2c). Sixty microarray proteins had statistically significantly higher antibody
179  magnitude in individuals with high-level TRA, after p-values had been adjusted to control the
180  false discovery rate (FDR) (Fig. 2e) [57]. The inverse, higher responses in individuals with no
181  TRA, was not observed (Supplementary Data 3). Logistic regression models with FDR
182  controlled p-values showed that antibody prevalence to 27 microarray targets was
183  significantly associated with high-level TRA, 25 of which were also significantly more
184  reactive in the analysis of signal intensity. Because large proteins were fragmented on the
185  array, the combined list of 62 novel microarray targets with TRA associated antibody
186  responses in either the linear (response intensity) or logistic (response prevalence) analysis
187  represented fragments of 43 unique proteins (Supplementary Data 2). 15 of the 43 proteins
188  with TRA-associated responses are conserved unknown proteins. Two are known
189  gametocyte/gamete proteins with putative roles in gamete egress; PF3D7_1038400
190  (gametocyte-specific protein, Pf11-1) and PF3D7_1449000 (gamete surface and sporozoite
191  traversal protein/GEST).

192  TRA-associated responses on the array showed only partial overlap with Pfs48/45 and
193  Pfs230 responses. Individuals with high-level TRA who were seronegative for both α-Pfs230
194  and α-Pfs48/45 (n = 10/22), had antibody responses to significantly more TRA-associated
195  microarray targets (median breadth 11 (out of 62) (interquartile range (IQR) 3–26)) than
196  gametocyte-positive individuals without TRA (n = 211; median breadth 4 (out of 62) (IQR 2–
197  9), LinR: $p = 0.018$). Responses to some TRA biomarkers were highly prevalent in
198  individuals with high-level TRA who lacked significant responses to Pfs45/45 and Pfs230
199  (e.g. Pf11-1 (PF3D7_103840) = 70% (n/N = 7/10); LSA3 (PF3D7_0220000) = 80% (n/N =

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8/10)). Responses to one target protein mapping to PF3D7_1103800 (CCR4-NOT) were present in 60% (n/N = 6/10) of high-level TRA α-Pfs48/45 and α-Pfs230 seronegatives, whereas seroprevalence for this target in individuals with no TRA was 10.3% (n/N = 26/254) (Supplementary Data 4).

Interestingly, 10 of the 43 proteins with TRA-associated antibody responses are putatively exported during early gametogenesis1. Though the majority of these proteins are enriched in asexual parasites, their association with TRA may reflect previous observations that TR immunity is induced and wanes rapidly after gametocyte exposure [26,27]. Responses to putative markers of asexual parasite exposure (PF3D7_1036000 (Merozoite surface protein 11 (MSP11)), PF3D7_0711700 (erythrocyte membrane protein 1, PfEMP1 (VAR)), PF3D7_0731600 (acyl-CoA synthetase (ACS5)), and PF3D7_0423700 (early-transcribed membrane protein 4 (ETRAMP4)) [51] were not associated with TRA, either in terms of response intensity (Bayes empirical t-test: FDR-adjusted p = 0.34–0.99) or seroprevalence (LogR: FDR-adjusted p = 1).

**TRA and mosquito infection rate in natural infections**. Next, we determined whether the immune profile associated with TRA in the SMFA was also associated with mosquito infection rates observed in direct membrane-feeding experiments. Data on the infectivity of donor blood samples to mosquitoes at the time of plasma donation was available for 494 individuals for whom gametocyte status had been assessed by microscopy and TRA assessed in the SMFA. Of the 128 individuals without patent gametocytaemia, 18 (14.1%) were infective to mosquitoes in the DMFA. Of the 366 gametocyte-positive individuals, 203 (55.5%) infected at least one mosquito, with a median mosquito infection rate of 20% (IQR 7.7–42.5%). There was good agreement between transmission outcomes in the SMFA using cultured gametocytes and *An. stephensi* mosquitoes, and the field-based DMFA using gametocytes in infected individuals and *An. gambiae* s.s. or *An. coluzzii* mosquitoes; Individuals with high-level TRA in the SMFA were significantly less likely to infect any mosquitoes in the DMFA (LogR: OR 0.23 (95% CI: 0.06–0.79), p = 0.020), and more likely to do so at a reduced rate (OR 0.10 (95% CI: 0.2–0.61), p = 0.012). The likelihood of gametocyte-positive individuals infecting any mosquitoes in the DMFA was significantly lower if seropositive for Pfs230 (LogR: OR 0.42 (95% CI: 0.22–0.78), p = 0.006), Pfs48/45 (OR 0.30 (95% CI: 0.16–0.59), p < 0.001), or either or both antigens (OR 0.33 (95% CI: 0.19–0.58), p < 0.001) (Fig. 3a). The proportion of mosquitoes that became infected after feeding was also significantly lower for individuals seropositive for Pfs230 (LogR: OR 0.36 (95% CI: 0.14–0.88), p < 0.027), Pfs48/45 (OR 0.14 (95% CI: 0.05–0.40), p < 0.001) or

either or both antigens (OR 0.25 (95% CI: 0.11–0.55), p = 0.001) (Fig. 3b). The proportion of mosquitoes that became infected decreased with increasing Pfs230 antibody density (LogR: OR for an increase of 0.1 normalised optical density 0.59 (95% CI: 0.40–0.86), p = 0.007) and Pfs48/45 antibody density (OR 0.43 (95% CI:0.28–0.66), p < 0.001).

Mosquito infection prevalence was lower in gametocyte positive individuals with antibodies recognising more of the 62 novel protein microarray targets with TRA-associated antibody responses (Supplementary Fig. 3). Individuals responding to ≥14/62 microarray proteins (14 being the 75th percentile of breadth of response to these targets) were significantly less likely to infect mosquitoes (LogR: OR 0.31 (95% CI: 0.18–0.51), p < 0.001), and more likely to do so at a reduced rate (OR 0.21 (95% CI: 0.09–0.45). p < 0.001) (Fig. 3a, b). After excluding Pfs48/45 or Pfs230 seropositive individuals, infectiousness and infection rate remained significantly lower in individuals responding to ≥14 of the 62 TRA-associated microarray proteins (infectiousness: LogR, OR 0.29 (95% CI: 0.16–0.53), p < 0.001, infection rate: OR 0.23 (95% CI: 0.10–0.52), p < 0.001).

**Functional involvement of antibodies in TRA.** To demonstrate whether naturally acquired antibodies to Pfs230 or Pfs48/45 were causally associated with TRA, we affinity-purified antibodies specific to key transmission-blocking epitopes of these proteins and assessed their activity in the SMFA (Table 2). These experiments were performed for 6 donors whose IgG showed high-level TRA and for whom high volumes of plasma (≥1 mL) were available; 3 of these donors had ≥10 mL plasma available, allowing additional testing after 9-fold antibody concentration of IgG. The flow-through of affinity purification experiments, depleted of α-Pfs230 and α-Pfs48/45 antibodies, was also tested in the SMFA to quantify TRA of antibodies to other target antigens. Concentrated back to the original plasma volume, α-Pfs48/45 antibodies of one donor independently inhibited transmission (TRA 91.5% (95% CI: 86.4–94.7)), whereas α-Pfs230 antibodies of a different donor had low but statistically significant TRA (TRA 41.1% (95% CI: 15.5–60.4)). When antibodies were concentrated to nine times their physiological concentration, α-Pfs48/45 antibodies from one additional individual, and α-Pfs230 antibodies from two additional individuals significantly reduced transmission to mosquitoes (Pfs48/45: TRA 81.3%, (95% CI: 70.9–88.0), Pfs230: TRA 94.8% (95% CI: 90.2–97.3) & 99.3% (95% 98.6–99.6), Table 2). For all individuals the α-Pfs48/45 and α-Pfs230-depleted IgG (containing no antibodies against Pfs48-45 epitopes 1–3, or Pfs230 epitope C, confirmed by ELISA) inhibited transmission to mosquitoes (Table 2). This activity was complement-independent.

269     To determine the presence of naturally acquired antibodies binding to unknown gamete

270     surface antigens, we performed surface immuno-fluorescence assays (SIFA) using gametes

271     of a Pfs48/45 knockout (KO) parasite [3] that does not produce surface retained Pfs23012.

272     Pfs48/45- and Pfs230-specific antibodies confirmed the absence of these proteins on

273     Pfs48/45 KO gamete surfaces (Fig. 4). The immuno-fluorescence of gamete surfaces

274     observed using total IgG and IgG depleted of α-Pfs48/45 and α- Pfs230 antibodies from

275     naturally exposed individuals therefore reflects recognition of unknown antigens on the

276     gamete surface (Fig. 4; Supplementary Fig. 5A).

277     Antibody responses to a number of the 43 novel proteins that are associated with TRA

278     (Supplementary Data 2) may be biomarkers of high prior gametocyte exposure rather than

279     causally related to TRA. We thus curated this list of proteins by excluding genes with known

280     or predicted intracellular products, while retaining genes based on evidence for a role in

281     transmission reduction or gamete viability, or predicted compatibility with

282     gametocyte/gamete surface expression. In addition to Pfs230 and Pfs48/45, 13 novel

283     proteins possess sequence characteristics that are compatible with surface expression or

284     have known roles in gamete viability (Table 3; Supplementary Data 5) and may thus form

285     biologically plausible targets of transmission-inhibiting antibodies. mAbs specific to Pf11-1,

286     one of the biomarkers with a role in gamete egress, were shown previously to reduce

287     transmission to mosquitoes in membrane-feeding experiments [58].

288     Previous data show that the *P. berghei* homologue of another of our markers,

289     PF3D7_1449000 (PfGEST), shows partial association with gamete surfaces and has a clear

290     role in gamete egress [59]. We tested three monoclonal antibodies (mAb) against PfGEST in

291     the SIFA and SMFA. SIFA with PfGEST mAbs showed no, or very limited surface reactivity,

292     indicating that PfGEST is not retained on the gamete surface by 20 min after erythrocyte

293     egress in *P. falciparum* (Supplementary Fig. 5B). Though two antibodies produced modest

294     TRA in singlicate SMFA experiments at 10 µg/mL (generalised linear mixed model (GLMM):

295     maximum 49.3% TRA, 95% CI: 31.0–62.8, $p < 0.001$), this was not replicable and showed

296     no improvement with concentration (Supplementary Fig. 4). These data indicate that though

297     naturally acquired PfGEST Ab appear to be biomarkers of TRA, there is no current evidence

298     to support a mechanistic role in TRA.

299     The 13 potentially surface expressed proteins were represented by 39 targets on the

300     microarray, of which 16 were statistically significantly associated with TRA in the SMFA.

301     Though antibody responses to most gametocyte proteins decrease with age, responses to

302     these 16 targets shared a pattern more similar to Pfs48/45 and Pfs230; breadth of response

303     to these targets increased with age ($p = 0.007$), whereas magnitude of response, though

highest in older individuals, showed no age-dependent association (Supplementary Fig. 6). Gametocyte-positive individuals recognising $\geq$ 4/16 of these targets (4 being the 75th percentile of breadth of response to these targets) showed lower infectiousness (LogR: OR 0.36 (95% CI: 0.23–0.56), p < 0.001) and infection rates (OR 0.32 (95% CI: 0.16–0.62, p = 0.001) in the DMFA (Fig. 3a, b). These patterns remained significant when Pfs48/45 or Pfs230 seropositive individuals were excluded (infectiousness: LogR, OR 0.34 (95% CI: 0.20–0.58), p < 0.001; infection rate: OR 0.31 (95% CI: 0.15–0.64), p = 0.001).

**Discussion**

In this study, we assessed antibody-mediated transmission reducing activity (TRA) in individuals naturally exposed to *P.falciparum* and identified associated antibody responses. A minority of samples (3.3%) demonstrated strong and reproducible levels of TRA in controlled in vitro assessments with cultured *P.falciparum* gametocytes; high-level TRA was associated variably with antibody responses to gametocyte antigens Pfs48/45, Pfs230 and 43 newly described proteins. These antibody responses were associated with reduced mosquito infection rates in feeding assays with local mosquito species on infected, gametocytaemic individuals. Although not all of the identified antibody responses will be causally involved in TRA, we provide the first direct evidence for independent roles of antibodies specific to Pfs48/45 and Pfs230 in naturally acquired TRA. The TRA of mAb specific to one of the novel TRA biomarkers, PfGEST, indicate that PfGEST antibodies elicited by *P. falciparum* exposure are likely to be non-functional biomarkers of TRA.

The transmission of *P. falciparum* to mosquitoes is influenced by numerous factors, including gametocyte density [60], sex ratio [61] and human host factors that are currently poorly characterised. A direct effect of naturally acquired antibodies on transmission efficiency is suggested by the observation that mosquito infection rates generally increase in mosquito-feeding experiments on naturally infected gametocyte carriers when autologous plasma is replaced by control serum [62]. Antibody responses to Pfs48/45 and Pfs230, established target antigens for transmission-blocking vaccines [15,36], are naturally acquired upon gametocyte exposure and have previously been statistically associated with TRA as measured in the SMFA [10,21–28]. The strength of these associations is variable and sometimes absent [29]; TRA is often observed in individuals without measurable α-Pfs48/45 or α-Pfs230 antibodies [10,23,25,28,30,31]. We performed SMFA experiments on purified IgG samples from 648 malaria-exposed donors and confirm previous associations between TRA and α-Pfs48/45 (OR 4.4, 95% CI: 1.5–12.9) and α-Pfs230 antibodies (OR 7.9, 95% CI: 2.8–22.8). Using a unique sample set with transmission data from DMFA from three endemic settings, we further provide the first evidence that α-Pfs48/45 and α-Pfs230 antibodies are

339 associated with reduced transmission efficiency during natural infections. Both the likelihood
340 of infecting any mosquitoes and the proportion of infected mosquitoes were significantly
341 lower for individuals with α-Pfs48/45 and α-Pfs230 antibodies. For the first time, we formally
342 demonstrate that naturally acquired antibodies to these gametocyte antigens can
343 independently inhibit mosquito infection after affinity purification.

344 Despite the important role of antibodies to these two prefertilisation antigens, 45% of plasma
345 donors with high-level TRA had no measurable α-Pfs48/45 or α-Pfs230 antibodies, and we
346 observed significant levels of TRA after IgG from a selection of donors was depleted of α-
347 Pfs48/45 and α-Pfs230 antibodies. Our affinity purification experiments were based on the
348 binding of antibodies to epitopes present in the Pfs48/45-10C and 230CMB vaccine
349 constructs, as the proteins' complex conformational structures so far preclude their
350 production as full-length proteins. We thus captured the most potent transmission-reducing
351 antibodies (Pfs48/45 epitopes 1–3 [21,22,36], Pfs230 epitopes within the C region [15]) but
352 not necessarily all antibodies to these proteins. Monoclonal antibodies against epitope 5,
353 which would not bind to the Pfs48/45-10C protein, have been associated with weak TR
354 activity [63]. However, given their lower efficacy and the immunodominance of epitopes 1–3
355 in naturally immune sera [21] it is highly unlikely that α-Pfs48/45 antibodies to epitopes other
356 than epitopes 1–3 would have contributed considerably to TRA. This is also supported by
357 the finding that antibodies to epitope 5 are only observed in the presence of antibodies to
358 epitopes 1–321. Because all known α-Pfs230 antibodies are complement dependent [64,65],
359 whereas TRA in the antibody-depleted fraction is complement independent, a role for
360 residual α-Pfs230 antibodies (responsive to epitopes outside the aa. 444–730 region of
361 Pfs230 represented by the 230CMB product) is similarly unlikely. Crucially, IgG depleted of
362 antibodies to Pfs48/45 epitopes 1–3 recognised surface antigens of a knockout parasite line
363 that fails to express Pfs48/45 and Pfs230, formally demonstrating recognition of other target
364 antigens by immune sera. Our findings of TRA in the absence of measurable α-Pfs48/45 or
365 α-Pfs230 antibodies, together with persisting recognition of gametes that do not express
366 these two proteins, and functional TRA after depleting IgG of antibodies to these two targets,
367 thus strongly suggest that responses to other antigens may contribute to naturally acquired
368 TRA.

369 Using a protein microarray, we aimed to identify whether responses to other gametocyte
370 targets were associated with TRA. This array was enriched for putative gametocyte surface
371 antigens but surface expression or gametocyte specificity were not prerequisites for
372 inclusion on the array. Our aim was to determine an immune signature of TRA that could
373 improve our understanding of transmission efficiency from natural infections. We therefore
374 included markers of gametocyte exposure as well as general markers of recent malaria

375   infection. We observed that responses to 43 proteins were statistically significantly
376   associated with high level antibody-mediated TRA. Responses to these antigens were
377   strongly predictive of transmission efficiency from natural *P.falciparum* infections with diverse
378   gametocyte strains and locally relevant mosquito species. Whilst further studies are needed
379   to assess the generalisability of this signature of TRA across endemic settings, the strong
380   association of our SMFA-based signature of TRA with reduced transmission potential during
381   natural infections is very promising. Future studies should aim to assess the kinetics of
382   antibody responses to these antigens and their association with TRA using longitudinal
383   cohorts, with well-defined clinical, parasitological and infectivity data.

384   The microarray proteins were produced in an *Escherichia coli* based in vitro transcription–
385   translation (IVTT) system. As conformation is not verified for all proteins, those requiring
386   posttranslational modifications will likely not have been expressed in their native
387   conformation. This was exemplified by Pfs48/45 that has a known complex structure [36,66]
388   and showed minimal reactivity or discriminative power when printed on the array as an IVTT
389   product (Supplementary Data 3). This apparent disadvantage of the IVTT system will
390   plausibly have affected our results for other conformational proteins, so the list of proteins in
391   Table 3 (and Supplementary Data 5) must be treated as a 'rule in' rather than 'rule out'
392   selection for proteins associated with antibody-mediated TRA. Among the proteins we
393   identified as having TRA-associated antibody responses, 13 novel proteins have sequence
394   characteristics suggestive of surface expression and may be targets of TRA. Previous data
395   indicate that two of the proteins found to be biomarkers of TRA in our analysis, PfGEST and
396   Pf11-1, were previously shown to have important roles in gamete egress from the
397   erythrocyte in mosquitoes [58,59]. Interestingly, Pf11-1 mAbs are able to reduce
398   transmission, possibly by disabling full gamete egress [58]. Our SIFA data with humanised
399   mAbs indicate that PfGEST has no clear surface expression in *P. falciparum* 20 min after
400   activation, which may explain their lack of replicable TRA in mosquito-feeding assays
401   compared to similar concentrations of α-Pfs230-specific mAb. As there is only a narrow
402   window of opportunity for α-Pf11-1 and α-PfGEST antibody binding during gamete egress,
403   activity against gamete surface proteins with roles in fertilization is a more biologically
404   plausible mode of antibody mediated TRA. Future experiments may identify which of the 13
405   other TRA-associated plausible antibody targets in Table 3 are expressed at the gamete
406   surface. Our findings identify the further characterisation of functional and non-functional
407   gametocyte antibody responses in individuals with naturally acquired TRA as a research
408   priority. Although the prevalence of high-level TRA decreases with age, an opposite trend is
409   observed for the magnitude and prevalence of antibodies to Pfs48/45 and Pfs230 and to
410   newly identified gametocyte antigens, in spite of their overall association with TRA

(Supplementary Fig. 6). A better characterisation of these antibody responses in relation to functional activity is needed and may include a detailed assessment of antibody isotype, affinity and complement-binding activities. Regardless of their functionality, our data indicate that possession of antibody responses to a group of *P. falciparum* proteins increases the likelihood of antibody-mediated transmission inhibition. Combined with assessment of α-Pfs48/45 and α-Pfs230, this signature of TR immunity may improve our understanding of natural malaria transmission dynamics.

**Methods**

**Study participants**. Serum was collected from individuals recruited to epidemiological studies in Burkina Faso, Cameroon and the Gambia (Table 1), as well as from Dutch migrants with a history of residence in P. falciparum malaria-endemic regions, who reported having been diagnosed with clinical malaria infection. Details of sample collection and recruitment criteria have been published for all samples from individuals living in endemic areas, but are provided here as a summary. Parasitological status was defined by microscopy acknowledging that this lacks the sensitivity to detect many low-density infections; i.e., microscopy negative samples were not treated as an unexposed control.

*Cameroon.* Samples (n = 140) were collected between 2011 and 2015 from individuals aged 5–16 years, during surveys at primary schools in the Mfou district, 30 km from Yaoundé. Data from these individuals have in part been presented previously [42,43], but the remainder were provided by Isabelle Morlais (IRD, Montpelier) without prior publication. Participants were primarily *P. falciparum* gametocyte carriers identified among asymptomatic children by microscopical screening. Gametocyte density (median (IQR) = 30 (16/80)/µL, among gametocyte positives) was calculated against 1000 leucocytes, assuming a density of 8000 leucocytes per µL.

*Gambia.* Samples (n = 226) were collected from individuals resident in Farafenni and nearby villages, approximately 200 km east of Banjul, in 1992–1994 [24,44], 1998 and 1999 [45], 2001 [46,47] and 2002 [48]. Individuals were aged 1–17 years at the point of sampling. Participants were enrolled if they were found to be asymptomatically infected with malaria parasites, based either on the observation of gametocytes [24,44], or asexual parasites [45–48] by microscopy. Gametocyte density (median (IQR) = 63 (10/390)/µL, among gametocyte positives) was either calculated by examination of 100 high powered fields, assuming that 1 gametocyte per field equated to 500/µL (threshold of 5 gametocytes/µL) as in Greenwood &

444 Armstrong 1991 [67], or by calculation against 1000 leucocytes, assuming a density of 8000

445 leucocytes per µL.

446 *Burkina Faso*. Samples were collected from residents in the villages of Laye and Dapélogo

447 (n = 192), ~30 km northeast and north of Ouagadougo, in the Central Sudan savannah area

448 [37,40,41], and from residents of Soumousso and Dande (n = 33), villages close to Bobo

449 Dioulasso [38,39]. The age of study participants ranged from 1.8 to 74 years. Recruitment

450 was either random [37,41], or based on the observation of gametocytes [38–40] or asexual

451 parasites [40] by microscopy. Gametocyte density (median (IQR) = 64 (32/128)/µL, among

452 gametocyte positives) was calculated against either 500 or 1000 leucocytes, assuming a

453 density of 8000 leucocytes per µL.

454 *Netherlands*. Dutch individuals were recruited by clinical staff at Radboud University Medical

455 Centre in Nijmegen, the Netherlands, and had either recently returned ( < 6 months) from

456 extended periods in endemic areas (n = 8) or had returned more than a year before

457 sampling (n = 49). Age ranged from 26–84 years. Those who had returned more than a year

458 before sampling had lived in areas with endemic *P. falciparum* transmission for longer than

459 15 years and reported between 2 and >80 clinical malaria episodes during that time. For

460 these individuals malaria infection history was only available through verbal testimony,

461 whereas 3 out of 8 of the recently returned migrants had confirmed recent or current malaria

462 infection. Samples were all collected between 1994 and 2014. As detailed previously, sera

463 from some of these expatriates show strong and consistent TRA in the SMFA [68].

464 All samples were collected after written informed consent was obtained from participants

465 and/or their parent(s) or guardian(s). Ethical clearance was provided by the National Ethics

466 Committee of Cameroon, the Gambian Government/Medical Research Council Joint Ethics

467 Committee, the Ethical Review Committee of the Ministry of Health of Burkina Faso and the

468 Centre MURAZ ethical review committees, the London School of Hygiene & Tropical

469 Medicine ethics committee and by the Radboud University Medical Center ethical committee.

470 **Sample preparation**. For SMFA, IgG was purified from all plasma samples using Protein G

471 HP Spintrap (GE Healthcare, GE Uppsala, Sweden), and concentrated to the original serum

472 volume using Vivaspin 20 centrifugal concentration columns (Sartorius AG, Goettingen,

473 Germany) following the manufacturer's instructions. Antibody purification was only attempted

474 for samples with sufficient serum volume for duplicate SMFAs (180 µL). For the serological

475 assays, an additional 30 µL of serum was retained if sample volume permitted. Because of

476 the high serum volumes necessary for the antibody depletion/purification experiments, these

477 were performed on only six samples with transmission-blocking antibodies. Three of these

478 six were processed by protein microarray. IgG for depletion/purification was purified from

479   1mL of serum using protein G high performance affinity columns (HiTrap™ Protein G HP 1
480   mL, GE Healthcare, Uppsala, Sweden), and then adjusted to the original serum volume for
481   testing in the specific Pfs48/45 and Pfs230 affinity columns using Vivaspin 20 centrifugal
482   concentration columns (Sartorius AG, Goettingen, Germany). Overall, 300 µL was
483   immediately removed for SMFA and serological assays, leaving 700 µL of total IgG at
484   physiological concentration for affinity column purification. Purification was repeated as
485   required to allow for antibody eluate concentration.

486   **Direct membrane-feeding assay (DMFA)**. DMFA was performed during the original studies
487   for 498/579 individuals from endemic areas, as described in the separate study protocols
488   using identical glass mini-feeders [54] (Coelen Glastechniek, Weldaad, the Netherlands) and
489   the same experimental protocol [69]. Of the 498 individuals with DMFA data, 494 had
490   microscopy-based estimates of asexual and gametocyte density; only gametocyte positives
491   (n = 366) were used in our DMFA analysis. All sites used colonies sourced from eggs caught
492   in local water sources. Colonies were of *A. gambiae* s.s. (>90%) (The Gambia [24,44–48]), *A.*
493   *coluzzii* (Cameroon [42,43] and Bobo Dioulasso in Burkina Faso [38,39]) or a comprised a
494   mixture of *A. coluzzii*, *A. gambiae* s.s. and hybrid forms (other Burkina Faso sites [37,40,41]).
495   Locally adapted mosquito colonies from all sites are available upon request. Data presented
496   in the current manuscript are the proportion of infectious individuals and percent of
497   mosquitoes infected with oocysts after feeding on whole blood [69].

498   **Standard membrane-feeding assay (SMFA)**. Mosquitoes used for the assessment of
499   antibody TRA in the SMFA were three to 5 day old female *Anopheles stephensi* (Sind-Kasur
500   Nijmegen strain) [70], which were reared at 30 °C and 70–80% humidity while exposed to a
501   12 h day/night cycle. Mature (stage IV-V) *P. falciparum* gametocytes (either NF54 [54], or
502   transgenic NF54HT-GFP-Luc [71], depending on the preferred readout) were obtained from
503   an automated tipper system (0.3–0.5% gametocytes, 2% haematocrit) and prepared with
504   packed red blood cells as previously described [9,54]. Both gametocyte strains can be
505   obtained from the MR4 Malaria Research and Reference Reagent Resource Center
506   (https://www.beiresources.org/MR4Home.aspx). For the preparation of mosquito blood
507   meals containing test antibodies, 90 µL of serum IgG was added to 90 µL of freeze-dried
508   foetal calf sera (FCS) and diluted into 35 µL human serum containing active complement.
509   This antibody/serum mix was added to the gametocyte/red blood cell mix to a final volume of
510   270 µL, and kept at 37 °C until it was provided to mosquitoes in the membrane-feeding
511   apparatus. Antibodies were therefore provided to mosquitoes at ~0.66* their physiological
512   concentration (assuming a 1:1 plasma/cell ratio). Each SMFA run was performed to test up
513   to 24 antibody samples simultaneously, including two control mosquito groups (against
514   which the TRA of test samples was calculated). For control samples 90 µL of freeze-dried

515 FCS was dissolved in 90 µL milliQ and added to the same gametocyte/red blood cell mix
516 (270 µL total volume) as was used for the other feeds the run.

517 Prior to blood feeding, male mosquitoes were separated from females by aspiration. Unfed
518 and partially fed female mosquitoes were removed from their resting containers after blood
519 feeding. Fully engorged mosquitoes were maintained at 26 °C and 70–80% humidity.
520 Mosquito infection status was determined differently for the NF54 and NF54HT-GFP-luc
521 parasite strains. For experiments with the native NF54 strain, an average of 19.5 mosquitoes
522 (range: 7–22) were dissected at day 7 post infection (PI). Midguts were stained in 1%
523 mercurochrome and oocysts were counted by expert microscopists. For experiments with
524 the transgenic NF54HT-GFP-luc strain, mosquitoes were killed on day 8 PI by freezing, and
525 20 were homogenised for each experiment in two pools of 10. Mosquito homogenates were
526 well mixed and three samples per pool were assayed for bioluminescence by lysis, and
527 addition of luciferase assay substrate using a Luciferase assay kit (Luciferase assay system
528 (E1501), Promega, USA), according to the manufacturer's instructions and as previously
529 described [9]. For experiments with the native NF54 strain, TRA was analysed as the
530 percent difference in oocyst number between test and control mosquitoes.

531 For experiments with the transgenic NF54HT-GFP-luc strain, TRA was calculated as the
532 average percent difference in luminescence intensity for two pools of 10 mosquitoes
533 between test and controls mosquitoes, as described9. TRA <0% was observed, but was
534 generally within the normal range due to natural variation in oocyst intensity between
535 mosquito groups. For individuals with TRA <0%, median TRA was -41.8% (IQR: −80.9–16.5).
536 For experiments with replication (e.g. the affinity purification experiments) 95% CI around the
537 TRA estimate were calculated from luminescence or microscopical readouts for multiple
538 feeds using generalised linear mixed models (GLMMs) [72]. Choice of readout (and
539 therefore of gametocyte strain) varied over the time that the SMFA's were being performed,
540 and makes no difference to the integrity or interpretation of TRA, only to its efficiency [9].
541 Overall, ⩾90% reduction in oocyst/luminescence intensity in test mosquitoes relative to
542 controls was classified as evidence of high-level TRA, which previous data indicate is highly
543 replicable [68]. Samples from all individuals with ⩾80% TRA were tested in duplicate to
544 ensure robustness of the functional phenotype and the mean value of duplicate experiments
545 was used to classify individuals as having high-level TRA (⩾90% average reduction) or no
546 evidence of TRA (<10% average reduction).

547 **Recombinant protein production for serological assays**. Recombinant, correctly folded
548 proteins were used for antibody depletion/purification experiments and for enzyme-linked
549 immunosorbent assays (ELISA). For all assays assessing Pfs230-specific antibodies, we

550  used the transmission-blocking vaccine candidate 230CMB (developed and purified by
551  Fraunhofer USA Center for Molecular Biotechnology), which corresponds to amino acids
552  444–730 of the Pfs230 protein, and comprises the protein's pro-domain and the first of
553  fourteen cysteine motif domains [15]. For assays with Pfs48/45, two protein constructs were
554  used. For antibody depletion/purification, we used the chimeric R0.10C protein [36], which
555  comprises 10 cysteines spanning epitopes 1–3 of the Pfs48/45 C-terminal domain, fused to
556  the GLURP R0 domain [36,40]. Antibodies specific to GLURP R0 have no effect on
557  Plasmodium transmission to mosquitoes (Supplementary Table 2) [9]. For non-functional
558  serologicalassays (ELISA, Microarray), the 10C protein was cleaved from the R0.10C as
559  described previously [40].

560  **Monoclonal antibodies**. SMFA data using mAb specific to GLURP R0, Pfs48/45
561  (85RF45.1), Pfs230 (2A2) and PfGEST are presented in Supplementary Fig. 4 and
562  Supplementary Table 2. Full details for GLURP R0 and Pfs48/45 (85RF45.1) mAb
563  production and SMFA is provided in Stone et al. [9] Details of Pfs230 mAb production and
564  SMFA are presented in Roeffen et al. 1995 [65]. PfGEST mAbs were produced by the
565  Hypothesis-driven Pre-erythrocytic Antigen Target Identification Consortium (HPATIC).
566  Briefly, HKL v2.0 KymiceTM containing human variable region genes (Kymab) were
567  immunised with recombinant protein expressed in the wheat germ cell-free system (CellFree
568  Sciences) adjuvanted with Montanide ISA 720. Non-antigen selected splenocytes were
569  sorted into 96 well plates, one cell/well, to generate single-cell mRNA material (Atreca).
570  Atreca's Immune Repertoire Capture (IRCTM) provides Single-Cell mRNA Barcode-
571  Sequencing with high throughput and accuracy. This platform captured full-length sequences,
572  enabling detection of somatic mutations across the entire antibody variable region. Natively
573  paired heavy and light chain IgG variable regions were analysed, and constant region
574  isotype assignments were made which were then produced via gene synthesis and
575  recombinant expression as fully human antibodies (LakePharma). Three mAbs were tested
576  in duplicate SMFA titration experiments. SMFA was conducted according to previously
577  published protocols [9]. In all mAb-based SMFA, the endpoint was oocyst quantification by
578  manual dissection.

579  **Pfs48/45 and Pfs230 antibody purification**. To purify antibodies specific to the Pfs48/45
580  and Pfs230 proteins from transmission-reducing sera, affinity columns were created by
581  coupling 1 mg of R0.10C or 230CMB to N-hydroxysuccinimide (NHS)-activated high
582  performance affinity chromatography columns (HiTrap™NHS-activated HP 1 mL, GE
583  Healthcare, Uppsala, Sweden); coupling efficiency was >80%.

584    For the extraction of antibodies from human serum, antibodies were extracted first using the

585    R0.10C column, and then using the 230CMB column, as follows. The R0.10C column was

586    equilibrated with 2mL of binding buffer (PBS (1×) before 700 µL of total IgG (at physiological

587    concentration, fraction 1 in Table 2) from transmission-blocking sera was added. The flow-

588    through was collected and the column washed with 12 mL of PBS. Overall, 5mL of elution

589    buffer (0.15M NaCl,0.1M glycine/HCL, pH 2.5) was applied to the column for the collection

590    for R0.10C-specific antibodies. After elution, the purification procedure was repeated using

591    the flow-through from the first purification, and the antibody eluates from both runs were

592    pooled. The final flow-through and combined antibody eluates (fraction 2a in Table 2) were

593    concentrated to 700 µL using Vivaspin 20 centrifugal concentration columns (Sartorius AG,

594    Goettingen, Germany). Overall, 300 µL of the flow-through was removed for SMFA (fraction

595    3 in Table 2) and serological assays, and the remaining 400 µL was processed over the

596    equilibrated 230CMB column as for the R0.10C column, except that the final flow-through's

597    (fraction 5 in Table 2) and eluates (fraction 4a in Table 2) were concentrated to 400 µL.

598    Depletion of specific antibody in the flow-through's of each column was confirmed with

599    ELISA. Antibodies (from eluates or flow-troughs) were processed in the SMFA in two

600    biological replicates, as for total IgG with >80% TRA in the larger SMFA screen. For three

601    samples, larger starting volumes were used to allow antibody eluates to be concentrated

602    nine times, to examine the effect of specific concentrated antibody on transmission-reducing

603    activity (fractions 2b and 4b in Table 2). The possibility that TRA from the column fractions

604    was due to cytokines or reactive ROx/RNOx species, which in circumstances of

605    inflammatory crisis can inhibit parasite development in mosquitoes [73], was excluded both

606    by our use of protein G binding columns for IgG purification for all samples, and our use of

607    30 kDa filters for antibody concentration prior to mosquito-feeding.

608

609    **Surface immuno-fluorescence assays (SIFA).** The Pfs48/45 KO NF54 *P. falciparum* line

610    was produced and characterised by van Dijk et al3. and subsequently assessed by Eksi et al.

611    [12] showed that Pfs230 is produced but not retained on surface of Pfs48/45 disruptant

612    gametes. Wild-type (WT) and Pfs48/45 KO gametocytes were generated using standard

613    protocols, as described above. Culture media Pfs48/45 KO line was supplemented with 2

614    µM pyrimethamine. Briefly, gametocytes were allowed to activate at room temperature for 20

615    min (for mAb SIFA) or 1 h (for human IgG SIFA) in the presence of foetal calf serum (FCS),

616    and gametes were prepared for microscopy by washing with PBS supplemented with 0.5%

617    FCS and 0.05% sodium azide. Antibodies were added to gamete preparations in the same

618    PBS/FCS buffer; mAbs at 5 µg/mL; human IgG/sera at dilutions of 1:20. Secondary

619    antibodies were Alexa Fluor TM 488 goat anti-human IgG (H+L), Alexa Fluor TM 488

620 chicken anti-mouse IgG (H+L), and Alexa Fluor TM 488 goat antirat IgG (H+L); all were

621 added at dilutions of 1:200. All gamete preparations were incubated with secondary antibody

622 at room temperature for 1 h.

623 **ELISA.** Antibodies specific to the Pfs48/45-10 C and Pfs230 (230CMB) proteins were

624 quantified in the ELISA exactly as described previously [40]. Optical density (OD) values

625 were normalised between assay plates by adjustment relative to a consistent point in the

626 linear portion of a standard curve of serially diluted highly reactive human sera. Cut-offs for

627 positivity were determined from normalised OD values using maximum likelihood methods to

628 define Gaussian populations of low and high responders as described previously [74]. For

629 Dutch migrants who had returned from endemic areas $\geq 1$ year before sampling, a

630 seropositivity threshold was calculated as the mean+3 standard deviations of the averaged

631 normalised OD values of eight naïve European control sera.

632 **Protein microarray.** The selection of proteins to be cloned and printed on the arrays was

633 made primarily based on observed expression by stage V *P. falciparum* gametocytes in a

634 single recent proteomic analysis [52]. To ensure that the array included most potential

635 antibody targets, we preferentially included proteins that had transmembrane domains,

636 signal peptides or GPI anchors. To ensure that the majority of proteins that were highly

637 abundant or 'specific' to mature gametocytes were included on the array, we used mass

638 spectrometry data from the analysis of the stage V gametocyte proteome [52], alongside

639 proteomic data from purified trophozoites [1] and schizonts (Supplementary Data 6, PRIDE

640 accession number: PXD008250) generated in the same laboratory. Gametocyte and asexual

641 stage specific expression data sets were generated in relative expression values to compare

642 protein abundances (label-free quantitative (LFQ) values) between the stages, which are

643 presented in Supplementary Data 1 as a fold-change value.

644 We chose to also include proteins that were potential markers of gametogenesis identified

645 by Silvestrini et al [1]. These proteins we identified as being expressed primarily by early

646 gametocytes, or shared between stages (proteins for 32/59 were present in the stage V

647 database), but some were more abundant in mature gametocytes or other parasite life

648 stages (Supplementary Data 1). In addition to these markers, we included four putative

649 markers of asexual parasite exposure: PF3D7_1036000 (Merozoite surface protein 11

650 (MSP11)), PF3D7_0711700 (erythrocyte membrane protein 1, PfEMP1 (VAR)),

651 PF3D7_0731600 (acyl-CoA synthetase (ACS5)), and PF3D7_0423700 (early-transcribed

652 membrane protein 4 (ETRAMP4)), based on work by Helb et al [51].

653 Details of the 315 proteins included on the array are given in Supplementary Data 1.
654 Proteins with reference sequences longer than 1000 amino acids were split into multiple
655 fragments (overlaps of at least 17 a.a) for cloning, in vitro protein expression and printing.
656 Because the basic criteria for the selection of proteins for microarray construction was based
657 on data generated from a single laboratory, we cross-referenced our protein list against a
658 cross-study analysis of gametocyte specificity [53]. In short, the specificity of any protein for
659 the gametocyte stage was scored by determining how often it had been detected across 11
660 proteomic analyses (listed in Supplementary Data 1: 3 of gametocytes only, 3 of asexual
661 stages only, and 5 of both asexuals and gametocytes). Proteins were binned from low to
662 high abundance and weighted according to the retrieval rates of proteins in two curated lists
663 of 'gold standard' gametocyte and asexual genes, consisting of genes that are known to be
664 specific for either asexual stages (n = 45) or gametocytes (n = 41). High expression of
665 gametocyte gold standard proteins with concurrent absence of nongametocyte gold standard
666 proteins resulted in a high gametocyte score, calculated from the fraction of retrieved
667 gametocyte genes over retrieved non-gametocyte genes. All scores were log-transformed
668 and summed over all data sets. Gametocyte scores were categorised using the scores'
669 distribution of the gametocyte gold standard as follows: scores above the first quartile (9.69)
670 were considered gametocyte-specific. Proteins scoring at least as high as the lowest scoring
671 gametocyte gold standard representative (-2.46) were considered as enriched in
672 gametocytes. Proteins with lower scores are not specific to a certain life stage and have
673 some evidence for expression in gametocytes. All proteins scoring lower than the median of
674 the asexual gold standard (-18.98) were considered asexual specific. This analysis
675 confirmed that 228 of the 315 proteins had consensus evidence for enrichment in
676 gametocytes (scores >−2.46), whereas 284/315 had conservative evidence for expression in
677 gametocytes (peptides present in at least one gametocyte proteomic database, score >−10).
678 312/315 were present in at least one gametocyte proteomic database, giving rise to a
679 score >−18.93. Scores for all proteins on the array are included in Supplementary Data 1. To
680 avoid further curation and potential bias, no proteins were excluded prior to analysis.

681 Proteins were expressed using an in vitro transcription and translation (IVTT) system, the
682 *Escherichia coli* cell-free Rapid Translation System (RTS) kit (5 Prime, Gaithersburg, MD,
683 USA). A library of partial or complete CDSs cloned into a T7 expression vector pXI has been
684 established at Antigen Discovery, Inc. (ADI, Irvine, CA, USA). This library was created
685 through an in vivo recombination cloning process with PCR-amplified CDSs, and a
686 complementary linearised expressed vector transformed into chemically competent E. coli
687 was amplified by PCR and cloned into pXI vector using a high-throughput PCR
688 recombination cloning method described elsewhere [50]. Each expressed protein includes a

689 5′ polyhistidine (HIS) epitope and 3′ hemagglutinin (HA) epitope. After expressing the
690 proteins according to manufacturer instructions, translated proteins were printed onto
691 nitrocellulose-coated glass AVID slides (Grace Bio-Labs, Inc., Bend, OR, USA) using an
692 Omni Grid Accent robotic microarray printer (Digilabs, Inc., Marlborough, MA, USA). Each
693 slide contained eight nitrocellulose 'pads' on which the full array was printed, allowing eight
694 samples to be probed per slide. Microarray chip printing and protein expression were quality
695 checked by probing random slides with anti-HIS and anti-HA monoclonal antibodies with
696 fluorescent labelling. Each chip contained 28 IVTT negative control targets (for data
697 normalisation), and 48 IgG positive control targets (for quality control).

698 For analysis of antibody reactivity on the protein microarray, serum samples were diluted
699 1:200 in a 3 mg mL−1 *E. coli* lysate solution in protein arraying buffer (Maine Manufacturing,
700 Sanford, ME, USA) and incubated at room temperature for 30 min. Chips were rehydrated in
701 blocking buffer for 30 min. Blocking buffer was removed, and chips were probed with pre-
702 incubated serum samples using sealed, fitted slide chambers to ensure no cross-
703 contamination of sample between pads. Chips were incubated overnight at 4°C with
704 agitation. Chips were washed five times with TBS-0.05% Tween 20, followed by incubation
705 with biotin-conjugated goat anti-human IgG (Jackson ImmunoResearch, West Grove, PA,
706 USA) diluted 1:200 in blocking buffer at room temperature. Chips were washed three times
707 with TBS-0.05% Tween 20, followed by incubation with streptavidin-conjugated SureLight P-
708 3 (Columbia Biosciences, Frederick, MD, USA) at room temperature protected from light.
709 Chips were washed three times with TBS-0.05% Tween 20, three times with TBS, and once
710 with water. Chips were air dried by centrifugation at 1000×g for 4 min and scanned on a
711 GenePix 4300A High-Resolution Microarray Scanner (Molecular Devices, Sunnyvale, CA,
712 USA). Target and background intensities were measured using an annotated grid file (.GAL).

713 Raw microarray protein target and local background fluorescence intensities, target
714 annotations and sample phenotypes were imported and merged in R (Foundation for
715 Statistical Computing, Vienna, Austria), where all subsequent procedures were performed.
716 Foreground target intensities were corrected for local background using the
717 backgroundCorrect function of the limma package [75]. Next, all corrected values were
718 transformed using the base 2 logarithm. The data set was normalised to remove systematic
719 effects by subtracting the median signal intensity of the IVTT controls for each sample. As
720 the IVTT control targets carry the chip, sample and batch-level systematic effects, but also
721 antibody background reactivity to the IVTT system, this procedure normalises the data and
722 provides a relative measure of the specific antibody binding to the non-specific antibody
723 binding to the IVTT controls (a.k.a. background). With the normalised data, a value of 0.0

724　means that the intensity is no different than the background, and a value of 1.0 indicates a
725　doubling with respect to background.

726　**Data analysis**. Quality control plots were made after each treatment of the microarray data,
727　which included boxplots and density plots of probe intensity by study sample and probe type.
728　Recombinant Pfs230 CMB was printed on the array for comparison to ELISA with the same
729　protein, yielding a correlation co-efficient (Spearman's rank) of 0.44 (p < 0.001). A universal
730　seropositivity threshold for reactivity to array proteins was established as the IVTT
731　background plus 3 standard deviations, which equated to a log2-transformed signal intensity
732　threshold of 0.92 (reactivity was therefore defined as approximately twice that of the
733　background). Because high-level transmission-reducing activity was uncommon in our
734　sample set (22/649 samples had TRA ≥90%), antigens were defined as 'reactive' if they
735　elicited seropositive responses in at least 1% of the study population (≥7 subjects), and
736　unreactive antigens were included in all analyses. The universal cut-off was used only for
737　defining proteins as reactive or unreactive, and for depicting antibody breadth for all
738　microarray proteins.

739　Statistical analysis was conducted using R (Foundation for Statistical Computing, Vienna,
740　Austria) or STATA 12 (StataCorp., TX, USA). The magnitude of antibody reactivity to each
741　antigen on the microarray by individuals in different groups was tested by empirical Bayes-
742　moderated t-tests [76], and by logistic regression after data binarisation using protein-
743　specific cut-offs; cut-offs were generated using maximum liklihoods methods (which
744　determines the junction between two Gaussian signal distributions for each protein that
745　proteins cut-off point for positivity) [74]. For the latter, proteins were dis-included from the
746　analyses if the mixture models failed to stably converge, or had to restart more than once. In
747　both the Bayes and logistic models, p-values were adjusted to control the false discovery
748　rate below 5% using the Benjamini–Hochberg (BH) method [57], and a finding was
749　considered significant for FDR controlled p-values (q-values) <0.05.

750　For analysis of TRA on a continuous scale, TRA was used as the log-transformed relative
751　intensity, to normalise the data and avoid compression at 0%. For clarity, figures presenting
752　TRA on a continuous scale present the linear TRA with a base of 0%. Linear models with
753　adjustment for gametocyte density (determined by microscopy, and given as
754　gametocytes/µL) were used to test for differences in continuous variables between groups.
755　Logistic models adjusted for gametocyte density were used to test for the association of
756　binary variables between groups, presenting odd-ratios and 95% CI. Differences in the
757　magnitude of response to all proteins by groups of individuals (in contrast to the Bayes
758　analysis, which compares responses to individual proteins by different groups of individuals)

759 were assessed with t-tests or ANOVA. Differences in mosquito infection prevalence in the
760 DMFA were analysed with logistic models adjusted for gametocyte density, while differences
761 in mosquito oocyst prevalence were assessed with logistic models, adjusted for gametocyte
762 density and with host (individual the mosquitoes were feeding upon) as a random effect. All
763 boxplots are standard Tukey's whisker and boxplots, with Tukey's method for outlier
764 determination. All DMFA-based analysis was performed on gametocyte-positive individuals
765 only. Protein-domain predictions for proteins with TRA-associated responses were made
766 using Interpro [77] or HhPred protein-domain prediction [78].

767 **Data availability.** The novel P. falciparum Schizont mass spectrometry proteomics data
768 have been deposited to the ProteomeXchange Consortium
769 (http://proteomecentral.proteomexchange.org) via the PRIDE partner repository [79] with the
770 data set identifier PXD008250. The authors declare that all other data supporting the
771 findings of this study are available within the article and its Supplementary Information, have
772 been deposited in the DRYAD data depository (https://doi.org/10.5061/dryad.8bp05), or are
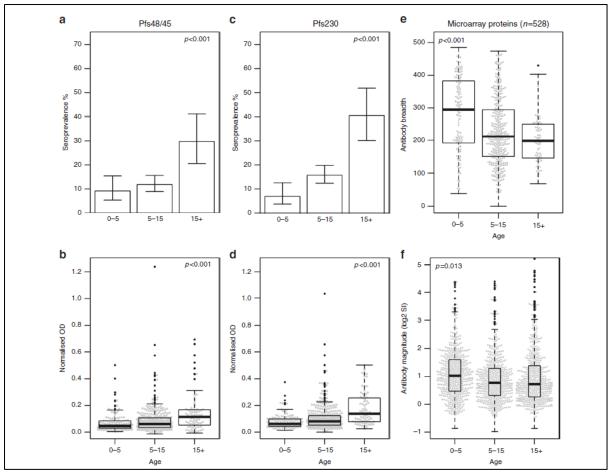773 available from the authors upon request.

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**Figure 1.** Antibody responses to Pfs48/45, Pfs230 and to the microarray proteins with age. Antibody responses to correctly folded, recombinant Pfs48/45- 10C and Pfs230 (230CMB) were measured using ELISA, with antibody intensity given as the ELISA optical density (OD) values (450 nm). Antibody responses to microarray proteins are given as the log2-transformed signal intensity (SI) minus the vehicle SI, which equates to the log2-fold change over this background. All graphs show only individuals from endemic areas (Dutch migrants excluded). Sample size: 0–5 = 131, 5–15 = 366, 15+ = 71. **a, c** Bars show the seroprevalence of α-Pfs48/45 and α-Pfs230 antibodies with age, with Clopper–Pearson confidence intervals. **b, d** Boxplots showing α-Pfs48/45 and α-Pfs230 antibody intensity with age. **e** Boxplots showing responses to the microarray protein targets (n = 528). Antibody breadth is the number of proteins reactive above background in each individual, within the given groups. **f** Magnitude of antibody response to microarray protein targets. Each dot represents the average SI of response to each protein target by all individuals within given groups. p-values for prevalence data are from likelihood ratio test for differences in seroprevalence between all age groups, derived from logistic regression and adjusted for gametocyte density. p-values for intensity data and response breadth are from an F-test for differences in OD/SI between all age groups, derived from linear regression and adjusted for gametocyte density or from ANOVA (for magnitude only). For all boxplots, outliers are shown in black, whereas all data points are shown in grey as a bee-swarm.

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**Figure. 2** Transmission-reducing activity (TRA) and antibody responses to Pfs48/45, Pfs230 and microarray proteins. TRA was categorised as described, to compare responses between gametocyte-positive individuals with <10% TRA, and individuals with >90% TRA. Antibody responses to correctly folded, recombinant Pfs48/45-10C and Pfs230 (230CMB) were measured using ELISA, with antibody intensity given as the ELISA optical density (OD) values (450 nm). Antibody responses to microarray proteins are given as the log2-transformed signal intensity (SI) minus the vehicle SI, which equates to the log2-fold change over this background. **a, b** Boxplots of α-Pfs48/45 and α-Pfs230 antibody intensity with TRA. **c** Responses to the microarray protein targets (n = 528). Antibody breadth is the number of proteins reactive above background in each individual, within the given groups. **d** Magnitude of antibody response to microarray protein targets. Each dot represents the average SI of response to each protein target by all individuals within given groups. p-values for intensity data and response breadth are from an F-test for differences in OD/SI between all age groups, derived from linear regression, and adjusted for gametocyte density or from students t-test (for magnitude only). For all boxplots, outliers are shown in black, whereas all data points are shown in grey as a bee-swarm. **e** Volcano plot showing the log2-fold change of the mean signal intensity for TRA as defined above. The p-value shown by the dotted line is unadjusted for false discovery. Black-circled data points are those that remain significant after p-values have been adjusted to control the rate of false discovery below 5%, using the Benjamini–Hochberg method.
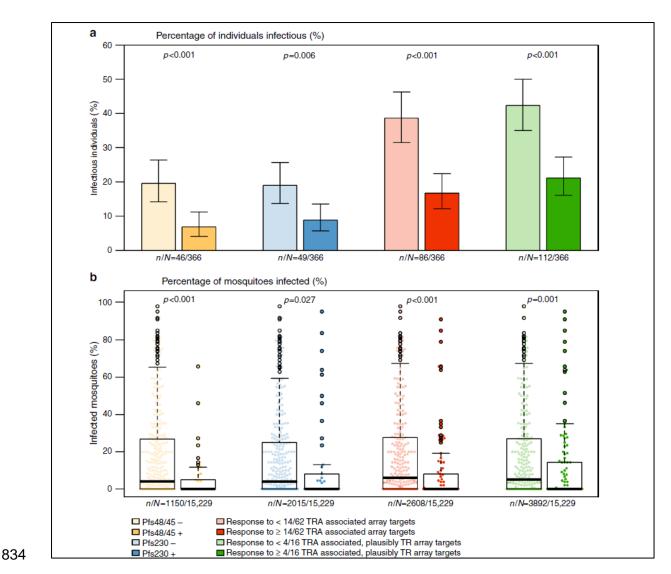
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**Figure. 3** Seroprevalence to Pfs48/45, Pfs230, and novel TRA-associated microarray proteins, and infectiousness in the direct membrane-feeding assay (DMFA). Individuals with DMFA data were categorised according to their possession of antibodies specific to: Pfs48/45 (positive (+)/negative (−)); Pfs230 (positive (+)/negative (−)); ≥14 of the 61 novel microarray proteins with TRA-associated antibody responses (14 being the 75th percentile of the breadth of response to these microarray targets among the entire sample set); ≥4 of the 16 novel microarray proteins with TRA-associated antibody responses that are also plausible targets of antibodies with TRA (4 being the 75th percentile of the breadth of response to these microarray targets among the entire sample set). **a** Bars show the proportion of infectious individuals among seropositive/seronegative gametocytaemic individuals with DMFA data, with Clopper–Pearson confidence intervals. n/N = number of individuals seropositive/total number of individuals with DMFA data. P-values are from logistic regression, with adjustment for gametocyte density. **b** Boxplots show the percentage of mosquitoes that became infected after feeding. n/N = the number of mosquitoes feeding on seropositive individuals/the total number of mosquitoes feeding on individuals with DMFA data. p-values are from logistic models, adjusted for gametocyte density and with host (individual the mosquitoes were feeding upon) as a random effect. For all boxplots, outliers are shown as hollow black circles, whereas all data points are shown as solid coloured circles.
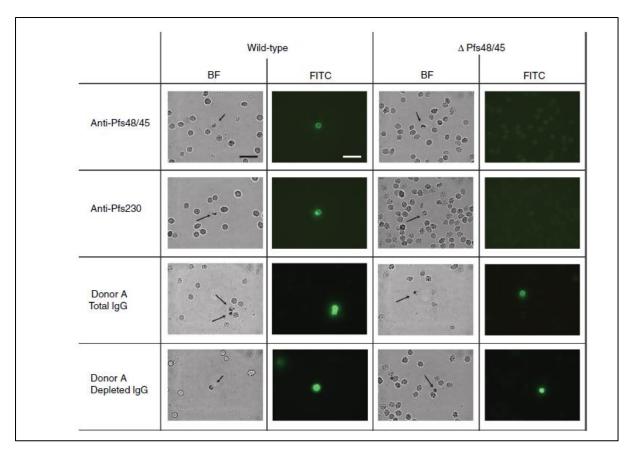
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**Figure. 4** Gamete surface immuno-fluorescence assay (SIFA) using wild-type and Pfs48/45 knockout (KO) NF54 gametes, with Pfs48/45 mAb, Pfs230 mAb, and IgG from a malaria-exposed serum donor. Donor IgG is from donor A in Table 2, and was performed using total IgG, and total IgG depleted of α-Pfs48/45-10c and α-Pfs230 (230CMB) IgG. Δ Pfs48/45 = Pfs48/45 KO3. BF Bright-field, FITC fluorescein isothiocyanate. Anti-Pfs48/45 is mAb 45.1, and anti-Pfs230 is mAb 2A2, as described in the methods. Scale bar is 20 µm.

**Table 1 Sample characteristics**

| Sample origin (study reference) | Samples (n) | Age (median, range) | Asexual positive (%) | Gametocyte-positive (%) | Parasite free (%) | Infectious (DMFA, %) | TR activity | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | <10% | ≥50% | ≥80% | ≥90% |
| Gambia[24, 44-48] | 226 | 5.0 (1.0–17.0) | 99.1% (224/226) | 74.2% (167/225) | 0.4% (1/226) | 24.0% (44/183) | 54.0% (122/226) | 15.9% (36/226) | 4.4% (10/226) | 3.1% (7/226) |
| Burkina Faso[24, 44-48] | 225 | 16.0 (1.8–74.0) | 56.5% (109/193) | 53.5% (114/213) | 31.9% (68/213) | 45.5% (86/189) | 68.4% (154/225) | 8.0% (18/225) | 2.7% (6/225) | 1.8% (4/225) |
| Cameroon[42, 43] | 140 | 8.5 (5.0–16.0) | 90.5% (117/133) | 93.5% (137/138) | 0.0% (0/139) | 73.8% (93/126) | 68.6% (96/140) | 14.3% (20/140) | 6.4% (9/140) | 4.3% (6/140) |
| The Netherlands[37–39, 43] | 57 | 79.5 (26.0–84.0) | 3.9% (2/51) | 3.9% (2/51) | 96.1% (49/51) | — | 57.9% (33/57) | 17.5% (10/57) | 8.8% (5/57) | 8.8% (5/57) |
| Combined | 649 | 7.1 (1–92) | 75.6% (458/606) | 65.8% (413/628) | 18.8% (118/629) | 44.8% (223/498) | 62.5% (405/648) | 13.0% (84/648) | 4.6% (30/648) | 3.4% (22/649) |

"—" data unavailable, or untested; *TR activity (%)*: percent transmission-reducing (TR) activity of purified IgG in the standard membrane-feeding assay (SMFA), relative to control mosquitoes fed the same gametocyte batch without test antibodies. TR activity is the mean of two independent SMFA runs for all samples with ≥80% TR activity in the first run, *Samples*: total number of samples with TR activity assessed in the SMFA, *Asex/Gct %*: asexual parasite and gametocyte prevalence by microscopy at the time of sampling, for individuals with available data, *Parasite free %*: no asexual parasites or gametocytes observed by microscopy

**Table 2 Activity of affinity-purified antibodies against R0.10C (Pfs48/45) and 230CMB (Pfs230) from transmission-reducing sera**

| ID | Location | Sex/age | Time since malaria exposure | TRA% (95% confidence intervals) | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | 1. Total IgG | 2a. R0.10C IgG | 2b. R0.10C IgG (conc.) | 3. Total IgG w/o R0.10c IgG | 4a. 230CMB IgG | 4b. 230CMB IgG (conc.) | 5a. Total IgG w/o R0.10C & 230CMB IgG | 5b. Total IgG w/o R0.10C & 230CMB IgG (C−) |
| A | Netherlands | M/69 | Infected | 99.4 (98.9–99.7) | 91.5 (86.4–94.7) | ND | 99.5 (99–99.7) | −2.5* (−38.6–24.2) | 99.3 (98.6–99.6) | 99.5 (99–99.8) | 99.8 (99.5–99.9) |
| B | Netherlands | — | Infected | 99.6 (99.3–99.8) | −42.8 (−80.5–12.9) | ND | 96.7 (95.6–97.5) | −15.7* (−46.1–8.4) | ND | 88.5 (85.7–90.7) | 87 (79.9–91.6) |
| C | Netherlands | — | <6 months | 99.9 (99.7–100) | 25.6* (−4.1–46.8) | 81.3 (70.9–88) | 99.4 (99–99.7) | 42.1 (15.5–60.4) | 94.8 (90.2–97.3) | 89.1 (80.2–94) | 95.8 (91.4–98) |
| D | Netherlands | M/74 | −5 years | 99.6 (99–99.8) | −14.7* (−65.3–20.5) | −0.3* (−38.7–27.4) | 85.4 (75.4–91.3) | 32* (−1–54.1) | 0.1* (−58.8–37.1) | 71.8 (52.7–83.2) | 80.8 (66.8–88.9) |
| E | Cameroon | F/12 | Infected | 98.8 (98–99.3) | 41.7 (26–54.1) | ND | 93.1 (90.3–95.1) | 16.5* (−8.6–35.8) | ND | 92.8 (89–95.3) | 93.4 (88.6–96.1) |
| F | Cameroon | F/6 | Infected | 100 (99.8–100) | 29.3* (−6.7–53.2) | ND | 99.9 (99.7–100) | 14.8* (−22.1–40.6) | ND | 97.2 (95.5–98.3) | 98.9 (96.7–99.6) |
| Ctrl | Netherlands | Pooled | Never | −26.5* (−72–7) | −17.2* (−50.9–9) | 4.6* (−33–31.5) | −14.7* (−50–12.3) | −6.3* (−31.4–14) | −2.7* (−41.5–25.5) | 6.5* (−19.4–26.9) | ND |

ND: not done. Transmission-reducing activity is from duplicate SMFA experiments, with luminescence intensity 7–9 days after mosquito-feeding (using NF54HT-GFP-luc strain gametocytes) as the output measure of infection intensity. All samples were tested in the presence of complement unless noted otherwise (C-). Total IgG represents the SMFA whole-purified patient IgG at physiological concentration. Pure IgG represents the TRA of antibodies that bound to the R0.10c and 230CMB columns. Conc. IgG represents pure antibodies at 9 times the physiological concentration. The fractions tested were as follows; 1. Total IgG, 2a. Purified α-R0.10c IgG, 2b. Concentrated (9×) α-R0.10C IgG, 3. Total IgG without α-R0.10c IgG, 4a. Purified α-230CMB IgG, 4b.Concentrated (9×) α-230CMB IgG, 5. Total IgG without α-R0.10C and α-230CMB-IgG. Fraction 5, depleted of antibodies binding to either protein column, was tested with and without (C-) complement. Ctrl is IgG from plasma from pooled naive Dutch donors. All TR values and confidence intervals were calculated from two independent SMFAs with different culture material. TRA, CI and p-values were calculated using generalised linear models (GLM) as described previously[72]. Asterisks (*) indicate results in which the oocyst/luminescence intensity of the test mosquitoes was not significantly different to the controls. Corrected ELISA OD values for α-10c IgG in flow-through fractions: 1. A = 0.12, B = 0.88, C = 0.15, D = 0.08, E = 0.13, F = 0.08, Ctrl = 0.02; 3. Range for all individuals 0.01–0.04. Corrected ELISA OD values for α-230CMB IgG in: 1. A = 0.05, B = 1.16, C = 0.76, D = 0.27, E = 0.10, F = 0.07, Ctrl = 0.06; 5. Range for all individuals 0.01–0.03

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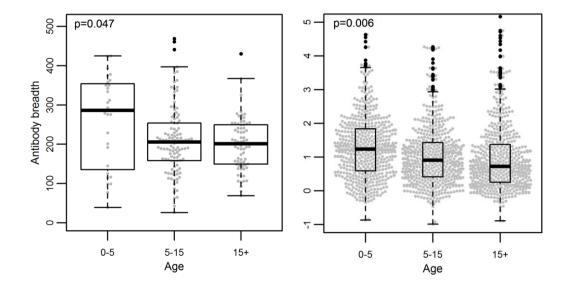975 natural isolates of gametocytes. *PLoS ONE* **10**, e0123777 (2015).

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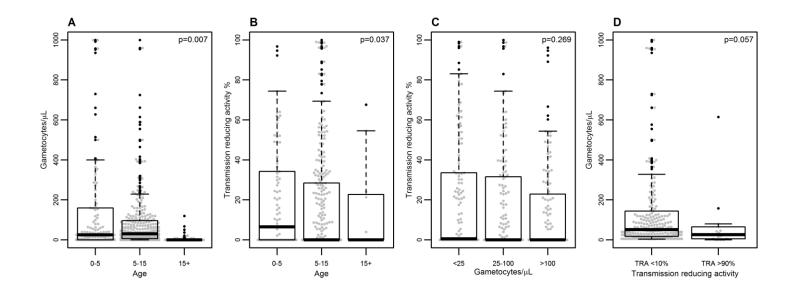1049    the standard membrane feeding assay. *Int. J. Parasitol.* **42**, 1037–1044 (2012).

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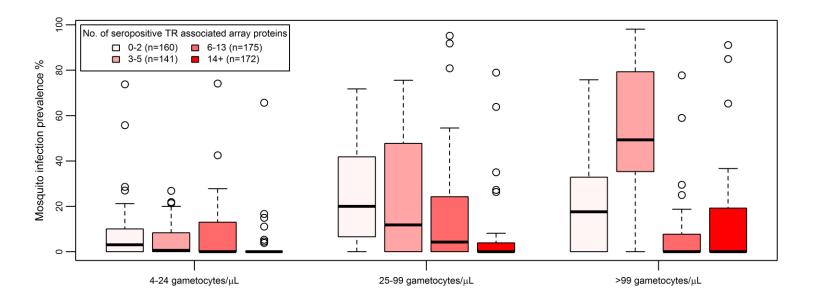
# Supplementary Information

**Supplementary Figure 1. Antibody responses to the gametocyte protein microarray, with age data for Burkina Faso only**. Responses by age are for individuals from Burkina Faso only, which was the only endemic country with substantial numbers of recruits from all age groups. Antibody breadth is the number of proteins reactive above background in each individual. Antibody magnitude is the log2-transformed average signal intensity (SI) for each microarray target, minus the vehicle SI. This equates to the log2-fold change over background. Responses are shown to each protein target by all individuals within given groups. For all box-plots, outliers are shown in black while all data points are shown in grey as a bee-swarm.
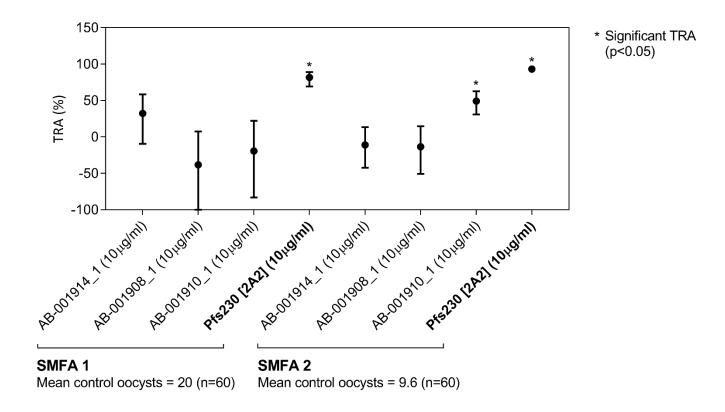
**Supplementary Figure 2. Gametocyte density and transmission reducing activity.** Gametocyte density and TRA over age (**A** & **B**) are for individual's living in endemic areas only. Gametocyte density is shown to 1000 gametocytes/µL in **A** and **D**. Values above this limit are excluded from the plots (points missing: 0-5=7, 5-15=12, 15+=0), but not the summary statistics for the box-plots (median, IQR). TRA on a continuous scale (**B** & **C**) is presented as absolute TRA for clarity, but the p values are from simple linear regression using log relative infectivity (log[oocysts in treatment mosquitoes/oocysts in control mosquitoes]), to normalise the data and avoid compression at zero. All plots and statistical analysis with age or gametocyte density include only individuals from endemic areas. Grouped TRA (**D**) is presented for individuals with antibodies causing TRA (TRA) ≥90% vs gametocyte positive individuals with antibodies causing TRA <10%, as presented elsewhere. For all box-plots, outliers are shown in black while all data points are shown in grey as a bee-swarm.

**Supplementary Figure 3. Mosquito infection prevalence decreases with breadth of response to novel TRA associated protein across all gametocyte densities.** Breadth of response to the 62 novel TRA associated protein targets from the array analysis were binned into quartiles among the population with DMFA data (n=366). Box plots show the median, 25th, 75th 5th and 95th percentiles of mosquito infection prevalence. For all box-plots, outliers are shown as hollow circles.

**Supplementary Figure 4. TRA of mAbs against Pfs48/45, Pfs230, and novel biomarker PfGEST.** For Pfs230 and PfGEST mAB, TRA was calculated against isotypic negative control mAb ($\alpha$-CSP). Pfs48/45 TRA was calculated using human serum as negative control. Details for Pfs48/45 (85RF45.1) mAb production and SMFA is provided in Stone et al. 2014 [1]. Details of Pfs230 mAb production and SMFA are presented in Roeffen et al. 1995 [2]. PfGEST mAb were produced by the Hypothesis-driven Pre-erythrocytic Antigen Target Identification Consortium (HPATIC) and provided by PATH. SMFA was conducted according to previously published protocols [1]. In all mAb based SMFA, the endpoint was oocyst quantification by manual dissection. Between 20 and 60 mosquitoes were dissected in each experiment. P-values are from a generalised linear mixed model (GLMM), as described previously [3].
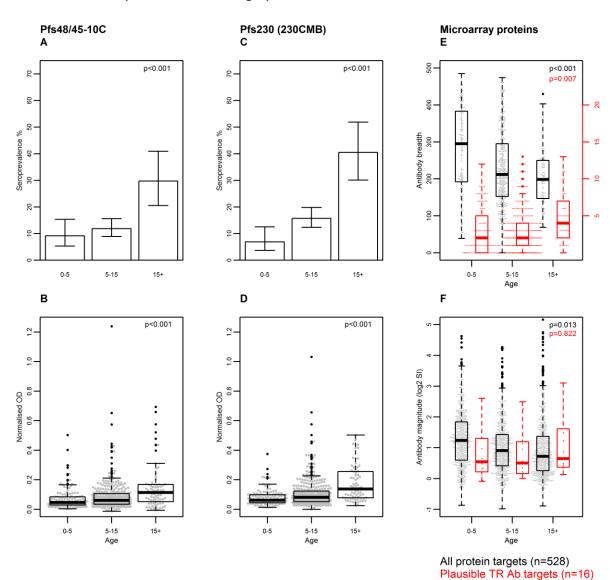
**Supplementary Figure 5. Gamete surface immuno-fluorescence assay (SIFA) using wild-type and Pfs48-45 KO NF54 gametes, with PfGEST mAb and IgG/plasma from a malaria exposed serum donor.** Donors B and E and naïve donor plasma are described in table 2. SIFA was performed with human antibody sources from whole plasma or total IgG depleted of α-Pfs48/45-10c and α-Pfs230CMB Ab. Δ Pfs48/45 = Pfs48/45 KO [4]. BF = Bright-field, FITC = fluorescein isothiocyanate. GEST mAbs were a mixture of 5µg/mL of all three GEST mAbs described in the methods. Scale bar is 20µm. **A.** Donor sera, **B.** mAb, with serum for comparison.

**Supplementary Figure 6. Figure 1. Antibody responses to Pfs48/45-10C, Pfs230 and to the microarray proteins with age, with microarray targets that are plausible antibody targets indicated.** Antibody responses to conformational, recombinant Pfs48/45 and Pfs230 were measured using ELISA, with antibody intensity given as the ELISA optical density (OD) values (450nm), which is normalised between assay plates by adjustment relative to a consistent point in the linear portion of a standard curve of serially diluted highly reactive human sera. Antibody responses to microarray proteins are given as the $\log_2$-transformed signal intensity (SI) minus the vehicle SI, which equates to the $\log_2$-fold change over this background. All graphs show only individuals from endemic areas (Dutch migrants excluded). Sample size: 0-5 = 131, 5-15 = 366, 15+ = 71. **A & C:** Bars show the seroprevalence of α-Pfs48/45-10C and α-Pfs230 antibodies with age, with Clopper-Pearson confidence intervals. **B & D:** Box plots showing α-Pfs48/45-10C and α-Pfs230 antibody intensity with age. **E:** Box-plots showing responses to the microarray proteins (n=528), and the targets which were identified as having characteristics which make surface expression or involvement in gamete viability possible (n=16). As indicated in the text, these 16 targets represent fragments of 13 unique proteins. Antibody breadth is the number of proteins reactive above background in each individual, within the given groups. **F:** Magnitude of antibody response to microarray proteins and plausible Ab targets. Each spot represents the average SI of response to each protein target by all individuals within given groups. P-values for prevalence data are from likelihood ratio test for differences in seroprevalence between all age groups, derived from logistic regression, and adjusted for gametocyte density. P values for intensity data and response breadth are from an F test for differences in OD/SI between all age groups, derived from linear regression, and adjusted for gametocyte density, or from students t-test (for magnitude only). For all box-plots, outliers are shown with larger black/red dots while all data points are shown in grey/red as a bee-swarm of small dots.

## Supplementary Tables

**Supplementary Table 1. Transmission-reducing activity and association with Pfs48/45 and Pfs230 antibody responses**

| SMFA selection | | Samples | Pfs230 (230CMB) | | | | Pfs48/45 (Pfs48/45-10C) | | | | Either/both of Pfs230 and Pfs48/45 | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | % (n/N) | OR (TRA >90%) | 95% CI | p-value | % (n/N) | OR (TRA>90%) | 95% CI | p-value | % (n/N) | OR (TRA>90%) | 95% CI | p-value |
| **Total** | **TRA (all)** | *648* | *16.2% (105/648)* | - | - | - | *14.4% (93/648)* | - | - | - | *21.8% (141/648)* | - | - | - |
| **Blockers** | **TRA ≥90%** | *22* | *50.0% (11/22)* | - | - | - | *40.9% (9/22)* | - | - | - | *54.5% (12/22)* | - | - | - |
| **Comparators** all samples | **TRA <90%** | 626 | 15.0% (94/626) | 5.8 | 2.2-15.8 | <0.001 | 13.4% (84/626) | 4.0 | 1.4-11.3 | 0.009 | 20.6% (129/626) | 5.1 | 1.9-14.0 | 0.001 |
| | **TRA <10%** | 405 | 15.6% (63/405) | 5.5 | 2.0-15.3 | 0.001 | 14.6% (59/405) | 3.7 | 1.3-10.5 | 0.015 | 22.0% (89/405) | 4.7 | 1.7-13.0 | 0.003 |
| **Comparators** gct positive only | **TRA <90%** | 406 | 12.1% (49/406) | 7.3 | 2.6-20.3 | <0.001 | 11.1% (45/406) | 4.7 | 1.6-13.6 | 0.004 | 17.2% (70/406) | 6.2 | 2.2-17.0 | <0.001 |
| | **TRA <10%** | 254 | 10.6% (27/254) | 8.2 | 2.9-23.7 | <0.001 | 11.4% (29/254) | 4.5 | 1.5-13.2 | 0.006 | 16.9% (43/254) | 6.2 | 2.19-17.45 | 0.001 |

**% (n/N)**  Seroprevalence of antibody responses to Pfs230, Pfs48/45, or either protein. n = seropositive individuals, N=total population size

**TRA %**  Transmission-reducing activity of purified IgG in the standard membrane-feeding assay (SMFA), relative to control mosquitoes fed the same gametocyte batch without test antibodies. TRA is the mean of two independent SMFA runs for all samples.

**Samples**  Total number of samples with TRA assessed in the SMFA

**n/N**  Number of samples seropositive in ELISA/Number of samples assessed in the ELISA

**OR (TRA ≥90%)**  Odds ratio of association of seropositivity and TRA, where the presence of TRA is defined as TRA ≥90%, and the absence of TRA is defined by the comparator groups TRA <90%, and <10%. Logistic models are adjusted for gametocyte density.

**95% CI**  95% confidence intervals of the OR

p-value  P-value from the logistic model used to generate the OR

**Supplementary Table 2. The TRA of GLURP-R0 compared to dilutions of mAb specific to Pfs48/45 epitope 1 (85RF45.1) and control human serum in the SMFA.** Full details of these SMFA experiments are in Stone et al. 2014[5].

| Treatment | SMFA 1 | | SMFA 2 | | SMFA 3 | |
|---|---|---|---|---|---|---|
| | Mean oocysts | TRA % | Mean oocysts | TRA % | Mean oocysts | TRA % |
| Control (human serum) | 6.8 | - | 13.3 | - | 17.9 | - |
| 85RF45.1 mAb (0.15µg/ml) | 6.3 | 7.2 | 11.2 | 15.8 | 30.7 | -71.4 |
| 85RF45.1 mAb (0.3µg/ml) | 2.9 | 57.0 | 13.6 | -2.5 | 16.1 | 10.2 |
| 85RF45.1 mAb (0.6µg/ml) | 1.4 | 79.8 | 7.5 | 43.7 | 9.2 | 48.7 |
| 85RF45.1 mAb (1.25µg/ml) | 0.1 | 98.0 | 1.2 | 90.7 | 1.9 | 89.4 |
| 85RF45.1 mAb (2.5µg/ml) | 0.1 | 99.0 | 0.4 | 96.7 | 0.3 | 98.5 |
| GLURP mAb (2.5µg/ml) | 6.9 | -2.2 | 14.1 | -6.0 | 12.1 | 32.5 |

**Supplementary References**

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