

1 ***Two faced immunity? The evidence for antibody enhancement of malaria***
2 ***transmission***

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11 Keywords: *Plasmodium*, Gametocytes, Gametes, Sexual stage immunity, Transmission
12 reducing/blocking immunity, Antibody dependent enhancement

13

14 **Abstract**

15 *Plasmodium* gametocytes can induce an immune response that interferes with the
16 development of sexual stage parasites in the mosquito gut. Many early studies of the
17 sexual stage immune response noted that mosquito infection could be enhanced as well
18 as reduced by immune sera. For *Plasmodium falciparum*, these reports are scarce, and
19 the phenomenon is generally regarded as a methodological
20 artefact. *Plasmodium* transmission enhancement (TE) remains contentious, but the
21 clinical development of transmission-blocking vaccines based on sexual stage antigens
22 requires that it is further studied. In this essay, we review the early literature on the
23 sexual stage immune response and transmission-modulating immunity. We discuss
24 hypotheses for the mechanism of TE, suggest experiments to prove or disprove its
25 existence, and discuss its possible implications.

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31 **Glossary**

32 **Transmission modulating immunity:** If antibodies targeting *Plasmodium* proteins
33 with a role in parasite development (e.g., Pfs48/45, Pfs230, and Pfs25) are ingested by
34 mosquitoes along with mature gametocytes in a blood-meal, antibody interaction can
35 prevent parasite development and cause mosquito transmission potential to be reduced
36 or blocked. As described in this review, these or other immune components may also
37 enhance immunity, by unknown mechanisms. Transmission modulating immunity may
38 be naturally acquired (see below, pre-fertilisation antigens) or elicited by vaccination.

39 **Mosquito feeding assay (MFA):** xenodiagnostic assay used to determine the
40 infectiousness of *Plasmodium* gametocytes to *Anopheles* mosquitoes. Mosquito feeding
41 assay may refer to skin feeding assays, in which mosquitoes are allowed to feed directly
42 on a subject's skin, **direct membrane feeding assays (DMFA)**, in which mosquitoes
43 feed on venous blood maintained at body temperature in a membrane feeding device, or
44 **standard membrane feeding assays (SMFA)**, in which mosquitoes feed on cultured
45 gametocytes in a membrane based feeder system.

46 **Transmission reducing activity (TRA)/% inhibition:** TRA is the percent inhibition of
47 infection (normally measured as the mean oocyst intensity) in a group of mosquitoes
48 under test conditions, relative to a group of mosquitoes under control conditions. Test
49 conditions may be the presence of a transmission reducing drug or antibody in the
50 infectious blood meal, while control conditions would indicate the absence of the
51 antibody in the same blood meal, or more properly the presence of an antibody which
52 has no effect on transmission.

53 **Relative infectivity:** An alternative metric to TRA/% inhibition for transmission data,
54 in which the mean oocyst intensity in test mosquitoes is presented as a value relative to
55 the mean oocyst intensity in control mosquitoes. TRA and relative infectivity are used
56 both used in the literature; in this review we favour the use of relative infectivity
57 (enhancement being positive, and reduction being negative).

58 **Gametocyte:** the sexual stages of the malaria parasite capable of reproduction in the
59 mosquito. Female and male gametocytes circulate in the human peripheral blood, where

60 they may be ingested by blood-feeding *Anopheles* mosquitoes and continue sexual
61 development.

62 **Gamete:** sexually dimorphic parasite forms that develop from gametocytes activating in
63 the mosquito gut to undergo fertilisation. Female gametocytes give rise to a single
64 female gamete, male gametocytes give rise to up to 8 motile microgametes; each female
65 gamete may be fertilised by a male microgamete.

66 **Pre-fertilisation antigen:** Antigens present during gametocyte development that are
67 retained during gamete formation and may have important roles in gamete fertility.
68 Naturally acquired transmission modulating immunity is due to exposure to pre-
69 fertilisation antigens including Pfs48/45 and Pfs230.

70 **Malaria transmission blocking vaccine (MTBV):** Vaccines designed to elicit
71 transmission reducing/blocking immunity in humans. MTBV may be based on antigens
72 present pre- and post-fertilisation, or non-*Plasmodium* antigens.

73

74 **Antibodies and *Plasmodium* transmission**

75 A dominant role of specific antibodies in controlling malaria disease severity was first
76 demonstrated in the 1960s by Cohen and McGregor [1, 2]. IgG from immune adults was
77 passively transferred to children with severe disease, rapidly reducing their parasite
78 density and improving their symptoms. Anti-*Plasmodium* antibodies have since been
79 shown to have multiple functions: preventing erythrocyte invasion by merozoites [3],
80 activating complement [4], stimulating neutrophil respiratory burst [5], opsonising
81 infected cells for phagocytosis [6, 7], reversing rosetting [6], preventing cells from
82 binding to the microvasculature [8, 9], and inhibiting sporozoite traversal or hepatocyte
83 invasion [10, 11]. Antibody responses against the transmissible gametocyte stages of
84 the parasite can also interrupt the parasites life cycle by preventing the parasites sexual
85 development in the mosquito midgut (**Box 1**). In short, the consequences of antibody
86 responses to *Plasmodium* parasites appear overwhelmingly disadvantageous for their
87 survival and transmission.

88 In other host-pathogen systems, parasite-antibody interactions may be more beneficial
89 to the pathogen. In 1964 Hawkes showed that highly diluted antibodies increased the
90 viral yields of flaviviruses including West Nile virus and Japanese encephalitis virus

91 [12]. Antibody dependent enhancement (ADE) of infection has since been observed *in*
92 *vitro* for many other viruses of medical and veterinary importance, including Dengue
93 virus (DENV), Human immunodeficiency virus (HIV), Zika Fever Virus, and foot-and-
94 mouth disease virus (FMDV) [12, 13]. Viruses with evidence for ADE share a few key
95 features: all replicate inside macrophages, all show a degree of antigenic diversity, and
96 all cause the production of partially neutralising antibodies [13]. For DENV,
97 enhancement has been linked with severe clinical consequences during secondary,
98 heterotypic infection in humans [12-18]. Halstead proposed that this was due to the
99 opsonisation of DENV particles by cross-reactive IgG, which would bind the virus to Fc
100 receptors on the macrophage surface, and possibly mediate immune suppression to
101 further increase viral load [19, 20].

102 For malaria parasites, there is sparse evidence of immune enhancement of asexual
103 parasite infection; monoclonal antibodies (mAb) to a *Plasmodium* asparagine rich
104 protein enhance invasion and growth of *in vitro* parasite cultures [21], and some
105 sporozoite specific antibodies, though inhibitory at high concentration, appear to
106 enhance hepatocyte invasion when diluted [22]. For sexual stage malaria parasites,
107 immune transmission enhancement (TE) is a common feature of the early literature in
108 both humans [23-29] and animals [30, 31]. In one of the most recent and
109 comprehensive assessments of transmission-modulating immunity in humans, standard
110 membrane feeding assays (SMFA) showed that a significant proportion (7%) of 642
111 immune sera from gametocyte positive individuals in Cameroon, Indonesia and
112 Tanzania enhanced the infectivity of gametocytes from culture by >20% [32].
113 Observations of antibody-mediated *Plasmodium* TE have been associated with low titres
114 of gamete-specific antibodies – while high titres are associated with the more
115 established and better quantified phenomenon of transmission-reduction (TR). An
116 untested hypothesis is that though low titres of anti-gamete antibodies may be unable
117 to reduce transmission, their binding to proteins present on both male and female
118 gametes may increase sexual interaction in the mosquito gut, increasing the likelihood
119 of successful fertilisation [24, 33].

120 Malaria control has entered a new era, in which declining global malaria incidence has
121 made elimination a realistic prospect, with vaccines targeting sexual stage parasites in
122 development as part of the intervention arsenal [34]. The consequences of naturally

123 acquired anti-gametocyte immunity for transmission efficiency are increasingly being
124 studied [35, 36]; TE as a possible counteracting immunological phenotype has not been
125 examined in recent years. Moreover, malaria transmission-blocking vaccines (TBV) are
126 currently being assessed in human volunteers [37], and trials with transmission or
127 incidence outcomes at the community level can be anticipated in the near future. As the
128 efficacy of malaria TBV's depends on the dynamics of the immune response to sexual
129 stage *Plasmodium* antigens, the evidence and potential mechanisms for antibody-
130 mediated *Plasmodium* TE, however equivocal, require re-examination.

131

132 **Assessing immune modulation of *Plasmodium* transmission**

133 Assessing immune modulation of transmission requires measurement of gametocyte
134 viability and infectiousness. *In vitro* assays can measure the interaction of immune
135 factors with intra-erythrocytic gametocytes [38, 39], and assess their inhibition of
136 gamete activity or the formation of post-zygotic parasites [40, 41]. The most
137 comprehensive assays for assessing transmission modulation are mosquito-feeding
138 assays, in which mosquitoes are allowed to feed on potentially infectious blood, and
139 transmission is later confirmed by the detection of *Plasmodium* oocysts in the mosquito
140 gut or sporozoites in the salivary glands. The blood source can either be from naturally
141 infected gametocyte carriers or non-malaria exposed donor blood mixed with
142 gametocytes from culture. In the former, transmission modulation by immune factors
143 can be demonstrated with direct membrane feeding assays (DMFA) by feeding
144 infectious blood to mosquitoes separately with the donors own (autologous) serum, or
145 with the serum of an individual with no exposure to malaria [42]; higher relative
146 infectivity with naïve serum would reflect serum mediated TR, while the opposite
147 would reflect TE [43, 44]. The standard membrane feeding assay (SMFA) with cultured
148 gametocytes allows for repeated measurements under controlled conditions [43], with
149 transmission modulation by added immune factors measured against controls fed the
150 same gametocyte-containing blood.

151 Using these assays, abundant evidence has accumulated that TR immunity exists in
152 *Plasmodium* exposed populations. Indirect evidence comes from studies showing that
153 mosquito infection rates tend to increase in the field-based DMFA when autologous
154 serum is replaced by naive serum [25-27, 45, 46]. The use of SMFA has formally

155 demonstrated that whole serum and (now more common) purified IgG from malaria-
156 exposed individuals can reduce mosquito infection rate and density [32, 47, 48]. The use
157 of purified IgG has the advantage that the transmission modulating effect of antibodies
158 of this class of immunoglobulins can be examined independent of other serum
159 components such as antimalarial drugs [49].

160

161 **Evidence for immune transmission-reduction and enhancement**

162 ***Animal models***

163 The existence of TR immunity was first definitively demonstrated in *Plasmodium*
164 *gallinaceum* infected chickens that had been immunised with inactivated gametocytes
165 or gametes [40, 50, 51]. Anti-gamete antibodies appeared to be to be short-lived, but
166 their titre was positively associated with gametocyte density and TR activity. Serum
167 from the immunised birds retained TR activity in mosquito feeding assays for 1-2
168 months, at which point monitoring ceased. Antibodies that bound gamete surfaces were
169 also observed in infected control birds immunised only with inactivated asexual stage
170 parasites, indicating *de novo* antibody generation in response to live sexual-stage
171 parasites [40, 51]. TR immunity was subsequently demonstrated by similar methods in
172 mice (*Plasmodium yoelli*) [52] and monkeys (*Plasmodium knowlesi*) [53, 54].

173 Inoculations with high densities of *P. knowlesi* microgametes stimulated long-lived TR
174 activity, which was successfully boosted by annual infection with blood stage parasites
175 and thus lasted the full 6 years of follow up in most animals [53].

176 Longitudinal observations of the immune response to viable infections were made from
177 Rhesus macaques infected with *Plasmodium cynomolgi* (a close relative of *Plasmodium*
178 *vivax*) [30]; **figure 1** is a graphic representation of anti-gamete antibody titres and
179 infectivity to mosquitoes during these infections. Anti-gamete indirect
180 immunofluorescence test (IFT) titres increased rapidly, in line with increasing parasite
181 density. Relative infectivity in the DMFA was highest prior to peak parasitaemia, when
182 anti-gamete titres were low and increasing. Peak parasitaemia coincided with the start
183 of a decline into TR activity, which was strongest between 11-19 days after patency,
184 when anti-gamete immuno-fluorescence test (IFT) titres peaked. As in chickens, anti-
185 gamete antibodies appeared to have short half-lives. In monkeys, enhancement was

186 again observed around 3 months after treatment during convalescence, when antibody
187 titres were similar to the pre-peak period (<1:320 reciprocal titre). The authors
188 reported that when total infectivity for each monkey was calculated as the sum of each
189 days mean oocyst count, 78-95% of the total infectivity between 0-150 days was during
190 a period when the animal's sera resulted in enhancement of transmission. In separate
191 experiments, transmission of *P. cynomolgi* from monkeys with prior *P. knowlesi*
192 infection was enhanced three-fold [31]. Here though, transmission modulation was not
193 attributable to serum factors; sera from monkeys previously infected had no enhancing
194 effect on gametocytes from monkeys with no prior infection.

195

196 ***Immune enhancement and reduction of transmission to mosquitoes in natural*** 197 ***infections in humans***

198 *Cross sectional assessments*

199 The first serological assessments of anti-gamete responses during naturally acquired
200 human infections showed evidence of serum mediated TR and TE [27]. Mendis et al.
201 showed that Sri Lankan patients with acute *P. vivax* infections produced antibodies that
202 bound *P. vivax* gamete proteins, and that their titre correlated with serum-mediated TR
203 activity in the DMFA. Notably, gametocytes from 3 of the 40 patients studied were less
204 infective to mosquitoes in the presence of naïve serum than autologous serum,
205 suggestive of TE.

206 In 1988, Graves et al. published the first direct evidence of TR immunity in humans
207 infected with *P. falciparum* [55], also demonstrating that malaria-exposed human sera
208 recognised sexual stage proteins Pfs230 and Pfs48/45 (**Box 2**). Among SMFA
209 experiments that were duplicated, enhancement of infection (131-204% of the control)
210 was observed in 6/33 individuals, the remainder showing variable levels of reduction
211 (0.6-89% of the control). These data from an area of intense transmission were
212 compared with an area of unstable transmission in Sri Lanka [25]. All Sri Lankan donors
213 were *P. falciparum* infected, and all infections were primary and symptomatic. TR
214 activity, assessed by serum replacement DMFA, was observed in 23/41 individuals,
215 while TE (relative infectivity between 125 and 400% of the controls) was observed in
216 13/41 individuals. Interestingly, immuno-precipitation of Pfs230 (in which the

217 fluorescent conjugate recognised IgG only) correlated poorly with TR activity, while
218 immuno-fluorescence assays (recognising IgG and IgM) correlated well.

219 In 1999 Healer et al. analysed TR immunity in 26 Gambian sera in SMFA experiments
220 [28]. Again, both reduction (5/26) and enhancement (7/26) were observed;
221 enhancement up to 10 times higher than control. High Pfs230 and Pfs48/45 Ab
222 reactivity was associated with low relative infectivity in the SMFA; low reactivity had no
223 clear association with infectivity. Importantly, both TR and TE were statistically
224 significant and reproducible.

225 Other analyses of sexual stage immunity with cross sectional or convenience sampling
226 have generally restricted their analyses to individuals with observable gametocytes by
227 microscopy. DMFA data from gametocyte carriers in high-endemic Yaoundé, Cameroon
228 showed that immune modulation occurred on a spectrum, with the majority of samples
229 showing some level of reduction. Among the 65 gametocyaemic donors TR (<50% of
230 the control oocyst intensity, referred to as 'high' reduction) was common (29/65 sera),
231 while very marginal higher infection (between 100-110% relative infectivity) was
232 observed in 7/65 donors. [29]. Justifiably, the latter was dismissed as evidence of
233 transmission enhancement. In DMFA experiments with serum replacement, the
234 transmission modulating effect of Cameroonian and Gambian sera was observed to vary
235 for autologous and non-autologous parasite isolates [56]. Of the 41 serum/isolate
236 combinations tested, 16 blocked and 2 enhanced transmission; both enhancing sera
237 blocked with different parasite isolates. Only one serum showed a consistent (blocking)
238 effect for all parasite isolates, indicating significant variability due to gametocyte
239 density, antibody titre, and/or antigenic polymorphism.

240 The most recent study with a specific focus on TE and TR immunity was by van der Kolk
241 [32], using 642 sera from patent *P. falciparum* gametocyte carriers in Cameroon,
242 Indonesia and Tanzania. The authors concluded that TR immunity was more common
243 than TE and had a larger effect size. Effect size was calculated as the relative
244 infectivity/the standard deviation of oocyst intensities; TR (effect size >0.2) was present
245 in 48% of sera, TE (effect size <0.2) in 7% of sera. Of 18 sera with TE in the primary
246 experiment, 6 (33%, p=0.01) retained their TE activity in a secondary feed. Of 175 sera
247 with TR, 101 (58%, p<0.001) retained TR in a second experiment. TR was associated
248 with anti Pfs48/45 and Pfs230 seropositivity whilst TE was not, i.e. individuals with

249 antibody titres over a defined cut-off were as common in the group that enhanced as in
250 the group that had no effect on transmission. A more informative analysis would have
251 assessed the association of specific antibody concentrations with ranked transmission
252 modulation.

253

254 *Longitudinal assessments*

255 A hypothesis that emerged from studies in animal models was that gamete antibodies
256 might have both TE and TR properties, which manifest according to their concentration
257 that varies over time (**Figure 1**)[30]. Such detailed assessments in humans may become
258 more viable with controlled human malaria infections allowing gametocyte production
259 [57, 58] but existing data from naturally acquired malaria infections inevitably start
260 from the point of patency or symptom presentation, excluding the assessment of
261 transmission-modulation early in the infection during antibody proliferation.

262 Among *P. vivax* patients sampled by Mendis and colleagues, six patients were followed
263 for 100 days after treatment and cure [26]. TR activity generally declined in line with
264 anti-gamete Ab titres, which had a half-life of around 2-months. However, by 80 days
265 post-treatment, serum from one individual was associated with TE 8 times higher than
266 the control. TR antibodies from these donors were later studied in the SMFA and
267 compared with parallel dilutions of anti-gamete mAb [24]. The results were
268 noteworthy: at high dilutions/low antibody concentrations, TR serum and mAb
269 promoted infection in mosquitoes feeding on blood that failed to infect mosquitoes in
270 their absence.

271 Various studies have assessed TR activity longitudinally but did not report TE. Non-
272 immune Javanese migrants arriving in Indonesian Papua acquired anti-gamete Ab and
273 TR immunity rapidly, and antibody titre appeared correlated with infection frequency
274 [59]. Assessments in Tanzania showed inconsistent patterns of TR activity with age, but
275 demonstrated the short-lived nature of sexual stage specific antibodies [60, 61]. The
276 object of these studies was specifically to examine immune TR, so relative infectivity in
277 the SMFA was capped at 100%, and TE was not reported.

278

279 *Monoclonal antibodies enhancing and reducing transmission to mosquitoes*

280 Monoclonal or polyclonal antibodies can be tested in DMFA or SMFA at a range of
281 dilutions, allowing assessment of the relationship between antibody titre and
282 transmission modulation. Most data available are for the transmission modulating effect
283 of P48/45 and P230 mAb.

284 Pieiris and colleagues showed that when transmission blocking *P. vivax* mAb (targeting
285 Pvs48/45) were diluted out in *P. vivax* gametocyte infected blood, the mAb TR activity
286 declined until at low titre they gave rise to enhanced transmission [24]. Diluted still
287 further, infection intensity returned to the same level as the control baseline. IgG
288 purified from the hybridoma supernatants showed the same effect. As for the human
289 sera from Sri Lanka described above, *vivax* specific mAb (diluted in naïve sera) were
290 able to promote infection in serum replacement DMFA experiments in which
291 gametocyte density was insufficient to cause infection alone.

292 Ponnudurai and colleagues investigated the impact of diluting *P. falciparum*
293 gametocytes densities and mAb concentrations independently [62]. Unexpectedly,
294 gametocyte dilution increased mosquito infection rate in the presence of anti-Pfs48/45
295 mAb, while decreasing infection rate in the presence of anti-Pfs25 mAb. This difference
296 may be due to increased fertilisation efficiency in parasites escaping reduction at low
297 Pfs48/45 antibody concentrations. When both mAb were diluted with static parasite
298 densities, relative infectivity initially declined, then enhanced by 19.1-23% at low titre
299 (0.01 – 0.02mg/ml), before returning to baseline infectivity at the lowest tested titre
300 (0.01-0mg/ml). This variation was judged to be '*within normal range*' relative to the
301 control, and therefore *in contrast to the enhancement of transmission by low antibody*
302 *concentrations observed s with P. vivax*'. These conclusions precipitated a view that
303 enhancement, if present, was lower in magnitude for *P. falciparum* than for other
304 species combinations.

305 A recent assessment aimed to compare SMFA outputs between two laboratories, using
306 the same mAb and human sera [63] (**Figure 2**). Pfs48/45 mAb (85RF45.1) caused
307 variable enhancement at the lowest tested concentration (1.2ug/mL) in one laboratory
308 (TropIQ, Netherlands), and variable reduction in the second lab at the same
309 concentration (LMVR, Bethesda, MD, USA). Further dilutions would be required to
310 clarify the effect of low 85RF45.1 mAb titres. On the other hand, IgG from human serum
311 caused enhancement at the lowest titre (23ug/mL) at both labs: this across 3 replicates

312 in each. Pfs25 mAb (4B7) caused no enhancement in either lab, but the lowest dilution
313 had not reached baseline in either laboratory.

314 Of note, mAb against central peptides of the D2 region of gametocyte/gamete protein
315 Pfs47 were recently shown to block transmission to mosquitoes, while mAb against
316 proteins at the N-terminus of the same region were shown to double the mean oocyst
317 density relative to controls [64]. These latest observations go against the hypothesis
318 that TE may be due to non-antibody components of immune sera.

319

320 **Testing immune transmission modulation and the mechanisms of action**

321 There are several reasons why historic evidence on the existence of immune-mediated
322 TE in needs to be interpreted with caution. **Box 3** summarises the uncertainties that
323 surround prior reporting on TE.

324 Despite these limitations, taken together previous assessments provide equivocal
325 evidence for *Plasmodium* TE, suggesting that low titres of antibodies in gametocyte
326 exposed individuals may enhance transmission, while high titres of the same antibodies
327 may reduce transmission (**Figure 2B**). Several possible mechanisms of action for TE
328 have been proposed. As gamete proteins are known to be present on both male and
329 female gametes (Pfs48/45 and Pfs230), enhancement could feasibly occur if antibodies
330 were able to bind simultaneously to proteins on both gamete sexes [24]. With IgG, the
331 presence of two binding sites makes this possible, though multiple gamete binding
332 would potentially be more effective with multi-meric IgM antibodies. Peiris and
333 colleagues suggested alternatively that enhancement may occur when low titres of
334 proteins critical to gamete fertilisation bind native protein, positively affecting protein
335 conformation, or that enhancement may be due to antibody mediated prevention of
336 inhibition by other human or mosquito factors [24]. The latter hypothesis would not be
337 unique to transmission stage parasites: Non-neutralising antibodies binding Merozoite
338 surface protein-1 (MSP-1) outside the MSP-1₁₉ region appear to compete with anti-
339 MSP-1₁₉ specific antibodies for its binding site during the parasites erythrocytic cycle.
340 Anti-MSP-1₁₉ antibody binding results in the inhibition of MSP processing, which is
341 required for cell invasion, whereas the binding of non-specific MSP antibodies results in
342 no such inhibition [65], thus enhancing infection rates.

343 De Arruda-Mayer suggested that TE of *P. cynomolgi* infection after exposure *P. knowlesi*
344 may be due to the absence of inhibitory serum factors during secondary infection rather
345 than the presence of enhancing factors, though they could not prove this [31]. Da et al.
346 showed that *Plasmodium berghei* infection was higher after dilution with uninfected
347 blood, despite the resulting decrease in parasite density [66]. It is therefore possible
348 that non-specific factors may contribute to transmission modulation (either the
349 presence of inhibitory factors during primary infections, or the absence of enhancing
350 factors).

351 Several experiments can be proposed to confirm the existence of transmission
352 enhancement and elucidate its mechanism (**Box 4**).

353

354 **Is malaria transmission enhancement relevant?**

355 As the sparse data described above suggests there is some degree of TE of for
356 *Plasmodium*, the obvious question is how this might impact broader transmission
357 dynamics. Modelling the impact of TE requires sensible parameterisation of its
358 frequency and magnitude, both of which are unknown.

359 ***Epidemiology***

360 When accurately quantified there appears to be a relatively simple, saturating
361 relationship between gametocyte density and mosquito infection rate [67]. In endemic
362 populations, gametocyte density is generally low and over-dispersed; surveys in Kenya,
363 Burkina Faso and the Gambia show that individuals who infect mosquitoes tend to
364 infect few (2-23% infection rate, with sample sizes between 19-97 mosquitoes) [68].
365 Based on the sparse evidence we have described, TE appears to have a lower effect size
366 than TR. However, as low gametocyte densities and low infection rates are the norm,
367 even small increases in mosquito receptivity to parasite development could significantly
368 affect population transmission potential. The relevance of intermediate TR activity on
369 controlled transmission between rodents has been demonstrated, warning against a
370 narrow focus on highly effective TR as the sole determinant of transmission efficiency
371 [69]. Similar experiments with antibodies causing low and intermediate TE would be
372 highly informative.

373 Few studies have aimed to link transmission-modulating immunity with natural
374 transmission rates in human populations. A recent study showed that high sexual stage
375 antibody titres were associated with significant transmission reduction in individuals
376 with high gametocyte burdens, but not in individuals with sub-microscopic infections
377 [35]. These assessments modelled the impact of specific antibody responses (Pfs48/45
378 and Pfs230) on natural infectivity in the DMFA. The absence of transmission inhibition
379 may be due only to the absence of reducing antibodies, but it is tempting to speculate
380 that enhancement may be apparent in some of these individuals. There is evidence from
381 longitudinal studies in Dielmo, Senegal that the efficiency of malaria transmission
382 increases as malaria is controlled. Between 1990 and 2007, slide prevalence of malaria
383 parasites decreased from 68 to 30%, while over the same period the proportion of
384 mosquitoes with sporozoites increased from 5 to 14% [70]. The increased transmission
385 was linked to higher gametocyte biomass in infected individuals, which could occur if
386 commitment rates were driven up by increased expression of the AP2-G protein [71].
387 The role of transmission modulating immunity was not considered, but it is possible
388 that the low antibody titres that result from infrequent parasite exposure (and thus
389 immune boosting) have enhanced the efficiency of transmission from infected
390 individuals gradually over time [70].

391 ***Vaccines***

392 Trials to evaluate the safety and immunogenicity of Pfs25 and Pfs230 based TBVs in
393 Malian adults are ongoing [37]. Such trials are welcome and long overdue, providing
394 hope that these or other candidate TBVs close to clinical assessment [72] may soon be
395 tested at the population level. If TE exists and is associated with low or waning antibody
396 titres, TBVs based on gametocyte proteins like Pfs230 could induce antibodies that
397 initially cause transmission blockade but may be followed by a period of TE. The
398 experiments suggested above will confirm if TE exists, and if it does, whether it is likely
399 to be induced by current TBV candidates, or instead by a response to alternative
400 epitopes within same protein, by a specific response to different (non-TR) proteins, or
401 by non-specific serum factors. In general, it is essential that the half-life of sexual stage
402 antibodies and the duration of their efficacy after exposure to natural gametocyte
403 antigens or TBVs be determined. It would also be prudent to ensure that individual
404 based studies assessing the longevity of immune response to TBV candidates in Phase I

405 and II trials continue follow up until and for a short time after antibody titres appear
406 return to baseline. Phase III trials, evaluated with transmission, infection or clinical
407 incidence outcomes, should incorporate longitudinal monitoring to rule out the possible
408 effects of TE, and assess the association of antibody titre with immune boosting by re-
409 infection.

410

411 **Concluding remarks**

412 We have known for decades that antibodies with specificity for gametocyte proteins can
413 inhibit *Plasmodium* establishment in the mosquito midgut. The knowledge that it could
414 work both ways, inhibiting and enhancing, could change our understanding of natural
415 malaria transmission and effect the development of vaccines based on sexual stage
416 proteins. At present, the evidence for TE in *P. falciparum* is incomplete whilst
417 comparatively more evidence exists for *P. vivax*. If TE is proven to occur, several
418 important questions will need to be answered to determine its relevance (see
419 Outstanding Questions). If TE effects are reproducibly observed in malaria exposed
420 human sera, it will be of significant interest to determine its mechanism and interpret
421 its role in natural malaria epidemiology; experiments to test its existence and
422 mechanism are suggested in **Box 4**. The potential induction of TE by TBVs will also need
423 to be investigated before it can be excluded.

424

425 **Funding**

426 WS, TB and CD are supported by the Bill & Melinda Gates Foundation (INDIE
427 OPP1173572). TB is further supported by a grant from the Netherlands Organization
428 for Scientific Research (Vidi fellowship NWO project number 016.158.306).

429

430 **Acknowledgments**

431 We would like to thank Geoff Targett for useful discussions on this topic, Kazutoyo
432 Miura and Koen Dechering for allowing us to present the data in figure 2, and the BMC
433 publishing group for allowing reproduction of this data.

434

435 **Conflicts of interest**

436 The authors declare that they have no conflicts of interest.

437

438 **Figure legends**

439 **Figure 1.** The relationship between anti-gamete antibody titre and infectivity to
440 mosquitoes during natural infection. **A.** Data from Naotunne et al. 1990 [30] showing
441 the relative infectivity of 4 toque monkeys (*Macaca sinica*) infected with *P. cynomolgi* to
442 *Anopheles tessellatus* mosquitoes. Relative infectivity was calculated as the geometric
443 mean oocysts in mosquitoes after a blood meal containing each monkey's own serum, as
444 a percentage of the geometric mean oocysts in mosquitoes after a blood meal in which
445 the monkey's serum was removed and replaced with naïve (from an uninfected monkey)
446 serum (*100). The infectious blood meal was centrifuged and washed before
447 resuspension in either autologous or non-immune sera. Reciprocal IFT titre is given as
448 reactivity to a gamete enriched mixture of *P. cynomolgi* parasites. **B.** Graphical
449 representation of the same data, with explanation of transmission modulating effects of
450 the anti-gamete antibodies.

451

452 **Figure 2.** Serial dilution of actual (**A**) and representative (**B**) transmission-blocking
453 human IgG in the standard membrane feeding assay (SMFA). **A.** Transmission inhibition
454 and titre of transmission blocking human IgG from a Dutch expatriate, who had lived for
455 many years in Cameroon and was gametocytaemic at the time of sampling (redrawn
456 from the original data of figure 4 of Miura et al. 2016 [doi: 10.1186] [63]). The sera were
457 tested in triplicate SMFA at two independent institutions; TropIQ (Nijmegen, the
458 Netherlands), and the Laboratory of Malaria and Vector Research (LMVR/ NIH,
459 Bethesda, MD, USA). Transmission inhibition (inhibition %) attributed to test antibodies
460 was calculated as the % inhibition of mean oocyst density relative to isotypic controls
461 (IgG from malaria naïve donors). Exact TR activity from replicates is denoted as F1/2/3.
462 Mix denotes the best estimate of the TR activity from the combined replicates, with 95%
463 confidence intervals (CI). SMFA was performed as described above, and full details are
464 in the paper in which these data were presented [63]. Average oocysts in the isotypic
465 control experiments of LMVR-F1, -F2, -F3, TropIQ-F1, -F2 and -F3 experiments were 3.9,
466 60.3, 14.0, 16.9, 4.3 and 5.9, respectively. **B.** Theoretical transmission reduction and

467 enhancement as a function of antibody titre, as might be apparent in a longer serial
468 dilution of the same antibody as in panel A. The Orange line represents IgG with
469 enhancing and reducing properties, the blue line represents IgG with only reducing
470 properties.

471

472 **Box 1. Immune responses to sexual stage *Plasmodium* sp.**

473 During their replication in the blood, a minority of *Plasmodium* schizonts become
474 committed to sexual development, producing merozoites that form gametocytes when
475 they invade healthy RBCs. *P. falciparum* gametocytes develop in the bone marrow, and
476 when almost mature are released back into the blood where they may be ingested by
477 blood feeding mosquitoes. The infectiousness of gametocytes to mosquitoes is
478 influenced by numerous factors, including gametocyte density [52-54, 73, 74], maturity
479 [75], sex-ratio [76], and human immune factors [77].

480 Human immunity may influence gametocyte transmission either by affecting
481 gametocyte formation and survival in the blood, or by affecting the life stages that
482 emerge in after ingestion by mosquitoes. There is some evidence that inflammatory
483 cytokines (TNF- α) may induce cell-mediated killing of asexual parasites and
484 gametocytes in hosts experiencing acute paroxysm [78, 79]. However, cell-mediated
485 gametocyte-specific killing in humans appears minimal or absent [80, 81]. Because
486 mature gametocytes lack the erythrocyte surface proteins of their asexual progenitors,
487 antibody responses targeting gametocyte-infected erythrocytes are also either absent
488 or difficult to detect [38, 39, 82, 83]. Eventually though, all gametocytes not transmitted
489 to mosquitoes break down in the blood, eliciting responses against gametocyte antigens
490 that are inaccessible to antibodies whilst gametocytes are circulating in the blood
491 stream. These gametocyte specific antibodies may be ingested by mosquitoes alongside
492 transmissible gametocytes, and if these antibodies interact with parasite proteins
493 involved in gametocyte activation or gamete fertilisation they may inhibit the parasites
494 further development in the mosquito. In this way, exposure to the sexual stages of
495 *Plasmodium* or to specific sexual stage antigens can induce transmission-modulating
496 (more commonly, transmission-reducing [TR]) immunity: an immunity elicited in the
497 blood, which functions only in the mosquito.

498

499 **Box 2. Pfs48/45 and Pfs230**

500 Early immunisation studies that stimulated interest in TR immunity [40, 50, 51] were
501 followed quickly by others that identified Pfs48/45 and Pfs230 as immuno-dominant
502 gamete surface proteins [84-86]. Monoclonal antibodies against Pfs48/45 protein are
503 able to bind and neutralise gametes and have potent transmission reducing activity in
504 mosquito feeding assays [84], whereas mAb specific to the larger Pfs230 lacked TR
505 activity in primary tests [84]. It was shown elsewhere that the TR activity of α -Pfs230
506 mAb was due to the antibodies activation of complement mediated gamete lysis [87-89].
507 The protein's presence in gametocytes is indicated by their recognition in malaria
508 endemic populations, and has been proven by proteomic analyses [90, 91].

509 Van Dijk et al. showed that Pfs48/45 was anchored to the gametocyte surface, and was
510 essential for fertilisation [92]. When Pfs48/45 was knocked-out, Pfs230 was not
511 observed on the gamete surface, indicating the protein was retained on the gamete
512 surface only by its association with Pfs48/45. On the other hand, targeted disruption of
513 Pfs230 also significantly inhibited oocyst production, indicating a central role in gamete
514 fertility, possibly in the formation of exflagellation centres by male gametes [93, 94].

515 Recognition of Pfs48/45 and Pfs230 in malaria exposed individuals is often but not
516 always associated with TR activity [28, 45, 59, 61, 77, 95]. This has led to an assumption
517 that other unknown gamete surface proteins may be jointly mechanistic in the
518 development of antibody responses with TR activity. Recent data show empirically that
519 naturally acquired human antibodies against Pfs48/45 and Pfs230 can reduce mosquito
520 transmission, independent of other serum antibodies [36], and that immune sera with
521 potent TR activity recognise unknown proteins on the surface of female gametes.
522 Antibody responses to proteins other than Pfs48/45 and Pfs230 are associated with TR
523 activity in the SMFA, and reduced transmission efficiency in the DMFA [36].

524

525 **Box 3. Factors influencing the reliability of observations of transmission**
526 **enhancement**

527 ***Assay performance***

- 528
- *The SMFA is optimised for assessment of strong transmission reduction*

529 The SMFA has been optimised to achieve consistently high oocyst intensity and
530 prevalence in control infections [96]. Though strong TR effects are detectable in
531 these ‘saturated’ conditions, TE may be masked. There are similar concerns that
532 because the SMFA does not produce naturalistic mosquito infections (ideally
533 with the majority of mosquitoes harbouring 1-5 oocysts [97, 98]), the assay may
534 not do justice to the effects of intermediate TR/TE activity [69].

- 535 • *The impact of non-specific factors in blood meals is unknown*

536 It is conceivable that higher non-specific antibody content in a blood meal may
537 be nutritive to parasites or mosquitoes, and that this could (directly or
538 indirectly) benefit parasite survival. Most previous assessments of immune
539 TR/TE have used isotypic controls to calculate relative infectivity (e.g. naïve
540 serum vs test serum, non-specific mAb vs TR mAb), but it has become
541 commonplace to use non-isotypic human or foetal bovine serum as a control for
542 feeds with additional purified antibodies, or m. If any transmission modulation is
543 due to non-specific blood meal components, the use of non-isotopic controls
544 could give rise to apparent TR/TE where there is none.

545 ***Reporting***

- 546 • *Transmission enhancement is not reported*

547 TE is often regarded as an artefact of the feeding system and not recorded.
548 Relative infectivity is often floored at 100% (i.e. 0% TR activity) in published
549 data. Artefact or otherwise, the true extent to which TE is observed is unlikely to
550 be fully reflected in the literature.

551 ***Experimental design***

- 552 • *Sample selection is biased toward transmission reduction*

553 The majority of studies have focused on infectivity or TR activity, sampling only
554 gametocyte positive individuals to boost infectiousness in the DMFA, or ‘to
555 increase the chances of observing anti-gamete responses’ [32]. Low sexual stage
556 antibody titre and TE may be most apparent at start and end of an infection, at
557 which times gametocytes are more likely to be sub-patent [99]. Indiscriminate
558 sampling or prospective longitudinal sampling may be more appropriate study
559 designs to capture the full range of immune transmission modulation.

560 • *Immune transmission modulation may vary between Plasmodium species*
561 Parasite species and strains are used interchangeably to provide evidence for
562 TE/TR, but differences in species gametocyte development may affect kinetics of
563 sexual stage immunity.

564 • *Is IgG purification appropriate for testing TE?*
565 Assessments of transmission modulation have focused on the impacts of total
566 IgG, but it is possible other antibody classes (e.g. IgM), sub-classes (e.g. IgG3), or
567 as above – non antibody factors may have different transmission modulating
568 properties, and that such effects are generally missed.

569

570 **Box 4. Considerations for testing *Plasmodium* transmission enhancement (TE)**

571 ***Does TE occur, and does it occur as a function of serum titre?***

572 To determine if TE occurs at low serum/Ab titre, dilution series SMFA (with serum,
573 purified serum Ab or mAb) should be conducted, ensuring that total antibody content is
574 consistent between feeds. Dilution should continue beyond the point at which relative
575 infectivity reaches 100% (TR activity 0%); if TE occurs at low titres, further dilution
576 would return infectivity to the level of the control (**Figure 2B**).

577 ***Is TE due to anti-gamete antibodies, or non-specific immune factors?***

578 SMFA could be conducted using whole sera, purified IgG (and other Ab isotypes), and
579 sera after extraction of antibodies to clarify the transmission-modulating effects of
580 antibody and non-antibody serum factors; controls should be isotypic i.e. SMFA with
581 whole endemic sera should use malaria naïve sera as controls.

582 ***Does TE occur with antibodies specifically elicited by TBV's?***

583 SMFA should include antibodies specific to both pre-fertilisation antigens (Pfs48/45
584 and Pfs230) and post-fertilisation antigens (e.g. Pfs25), to investigate mechanisms other
585 than enhancement of gamete fertilisation (e.g. enhanced midgut homing/binding by
586 ookinetes). SMFA should be conducted with and without complement; though some
587 sexual stage antibodies (α -Pfs230) are known to have complement mediated TR activity
588 [87] it is unclear whether the mechanisms leading to enhancement would be similarly
589 dependent. Experiments should also include both functional (blocking) and non-

590 functional mAb, as it is currently unclear whether TE is due to Ab binding to TR
591 epitopes, distinct non TR epitopes, or whether any gamete binding is sufficient [64].

592 ***Is TE due to binding antigens on adjacent gametes?***

593 This hypothesis could be tested with bi-specific antibodies; one fab region targeting a
594 gamete antigen, the other targeting a non-malaria specific antigen (e.g. an HIV protein).
595 If the presence of two binding sites is responsible for enhancement with IgG, dilution of
596 bi-specific antibodies will result in a linear decline of TR activity with Ab titre, while
597 mono-specific antibodies will cause enhancement at lower titres [100].

598 ***Do different antibody classes/sub-classes modulate transmission differently?***

599 IgM has more binding sites than IgG, which increases the likelihood of binding different
600 gametes. Each bond may have lower affinity, but multiple binding may result in a net
601 increase in avidity. Purification of IgM from immune sera for the SMFA is therefore of
602 significant interest for the assessment of transmission modulation. As antibody
603 concentration, affinity, circulation time, and complement activating activity could
604 feasibly affect transmission modulating activity [88], assessments focused on antibody
605 sub-class would also be valuable.

606

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