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

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

Abstract

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

Glossary

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

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

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

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

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

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

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

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

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

Antibodies and *Plasmodium* transmission

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

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

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

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

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

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

Assessing immune modulation of *Plasmodium* transmission

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

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

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

Evidence for immune transmission-reduction and enhancement

Animal models

The existence of TR immunity was first definitively demonstrated in *Plasmodium gallinaceum* infected chickens that had been immunised with inactivated gametocytes or gametes [40, 50, 51]. Anti-gamete antibodies appeared to be to be short-lived, but their titre was positively associated with gametocyte density and TR activity. Serum from the immunised birds retained TR activity in mosquito feeding assays for 1-2 months, at which point monitoring ceased. Antibodies that bound gamete surfaces were also observed in infected control birds immunised only with inactivated asexual stage parasites, indicating *de novo* antibody generation in response to live sexual-stage parasites [40, 51]. TR immunity was subsequently demonstrated by similar methods in mice (*Plasmodium yoelli*) [52] and monkeys (*Plasmodium knowlesi*) [53, 54]. Inoculations with high densities of *P. knowlesi* microgametes stimulated long-lived TR activity, which was successfully boosted by annual infection with blood stage parasites and thus lasted the full 6 years of follow up in most animals [53].

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

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

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

Cross sectional assessments

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

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

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

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

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

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

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

Longitudinal assessments

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

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

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

Monoclonal antibodies enhancing and reducing transmission to mosquitoes

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

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

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

A recent assessment aimed to compare SMFA outputs between two laboratories, using the same mAb and human sera [63] (**Figure 2**). Pfs48/45 mAb (85RF45.1) caused variable enhancement at the lowest tested concentration (1.2ug/mL) in one laboratory (TropIQ, Netherlands), and variable reduction in the second lab at the same concentration (LMVR, Bethesda, MD, USA). Further dilutions would be required to clarify the effect of low 85RF45.1 mAb titres. On the other hand, IgG from human serum 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-1_{19} region appear to compete with anti-
339 MSP-1_{19} specific antibodies for its binding site during the parasites erythrocytic cycle.
340 Anti-MSP-1_{19} 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.

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

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

Is malaria transmission enhancement relevant?

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

Epidemiology

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

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

Vaccines

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

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

Concluding remarks

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

Funding

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

Acknowledgments

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

Conflicts of interest

The authors declare that they have no conflicts of interest.

Figure legends

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

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

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

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

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

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

Box 2. Pfs48/45 and Pfs230

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

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

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

Box 3. Factors influencing the reliability of observations of transmission enhancement

Assay performance

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

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

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

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

Reporting

- *Transmission enhancement is not reported*

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

Experimental design

- *Sample selection is biased toward transmission reduction*

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

- *Immune transmission modulation may vary between Plasmodium species*

  Parasite species and strains are used interchangeably to provide evidence for TE/TR, but differences in species gametocyte development may affect kinetics of sexual stage immunity.

- *Is IgG purification appropriate for testing TE?*

  Assessments of transmission modulation have focused on the impacts of total IgG, but it is possible other antibody classes (e.g. IgM), sub-classes (e.g. IgG3), or as above – non antibody factors may have different transmission modulating properties, and that such effects are generally missed.

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

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

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

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

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

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

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

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

Is TE due to binding antigens on adjacent gametes?

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

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

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

References

1. McGregor, I.A. et al. (1963) Treatment of East African P. falciparum malaria with West African human γ-globulin. Trans. R. Soc. Trop. Med. Hyg. 57 (3), 170-175.
2. Cohen, S. et al. (1961) Gamma-Globulin and Acquired Immunity to Human Malaria. Nature 192 (4804), 733-737.
3. Blackman, M.J. et al. (1990) A single fragment of a malaria merozoite surface protein remains on the parasite during red cell invasion and is the target of invasion-inhibiting antibodies. The Journal of Experimental Medicine 172 (1), 379-382.
4. Boyle, M.J. et al. (2015) Human antibodies fix complement to inhibit Plasmodium falciparum invasion of erythrocytes and are associated with protection against malaria. Immunity 42 (3), 580-590.
5. Perraut, R. et al., Association of antibody responses to the conserved Plasmodium falciparum merozoite surface protein 5 with protection against clinical malaria, PloS one, 2014, p. e101737.
6. Ghumra, A. et al., Immunisation with recombinant PfEMP1 domains elicits functional rosette-inhibiting and phagocytosis-inducing antibodies to Plasmodium falciparum, PloS one, 2011, p. e16414.
7. Lee, S.H. et al. (1989) Antibody-dependent red cell removal during P. falciparum malaria: the clearance of red cells sensitized with an IgG anti-D. Br J Haematol 73 (3), 396-402.
8. Turner, L. et al. (2013) Severe malaria is associated with parasite binding to endothelial protein C receptor. Nature 498 (7455), 502-505.

9. Nielsen, M.A. et al. (2009) Induction of adhesion-inhibitory antibodies against placental Plasmodium falciparum parasites by using single domains of VAR2CSA. Infection and immunity 77 (6), 2482-2487.
10. Roestenberg , M. et al. (2009) Protection against a Malaria Challenge by Sporozoite Inoculation. N Engl J Med 361 (5), 468-477.
11. Nussenzweig, R.S. et al. (1967) Protective Immunity produced by the Injection of X-irradiated Sporozoites of Plasmodium berghei. Nature 216 (5111), 160-162.
12. Hawkes, R.A. (1964) Enhancement of the infectivity of arboviruses by specific antisera produced in domestic fowls. Aust J Exp Biol Med 42 (4), 465-482.
13. Tirado, S.M.C. and Yoon, K.-J. (2003) Antibody-Dependent Enhancement of Virus Infection and Disease. Viral Immunol 16 (1), 69-86.
14. Halstead, S.B. (1970) Observations related to pathogensis of dengue hemorrhagic fever. VI. Hypotheses and discussion. The Yale journal of biology and medicine 42 (5), 350-362.
15. Halstead, S.B. (1979) In Vivo Enhancement of Dengue Virus Infection in Rhesus Monkeys by Passively Transferred Antibody. The Journal of Infectious Diseases 140 (4), 527-533.
16. Vaughn, D.W. et al. (2000) Dengue Viremia Titer, Antibody Response Pattern, and Virus Serotype Correlate with Disease Severity. The Journal of Infectious Diseases 181 (1), 2-9.
17. Halstead, S.B. et al. (1970) Observations related to pathogenesis of dengue hemorrhagic fever. IV. Relation of disease severity to antibody response and virus recovered. The Yale Journal of Biology and Medicine 42 (5), 311-328.
18. Sangkawibha, N. et al. (1984) Risk factors in dengue shock syndrome: A prospective epidemiologic study in Rayong, Thailand. The 1980 outbreak. Am J Epidemiol 120 (5), 653-669.
19. Halstead, S.B. and O'Rourke, E.J. (1977) Dengue viruses and mononuclear phagocytes. I. Infection enhancement by non-neutralizing antibody. The Journal of experimental medicine 146 (1), 201-217.
20. Halstead, S.B. et al. (2010) Intrinsic antibody-dependent enhancement of microbial infection in macrophages: disease regulation by immune complexes. Lancet Infect Dis. 10 (10), 712-722.
21. Franzén, L. et al. (1989) Enhancement or inhibition of Plasmodium falciparum erythrocyte reinvasion in vitro by antibodies to an asparagine rich protein. Mol Biochem Parasitol 32 (2), 201-211.
22. Nudelman, S. et al. (1989) Dual action of anti-sporozoite antibodies in vitro. The Journal of Immunology 143 (3), 996.
23. Graves, P.M. et al. (1988) Measurement of malarial infectivity of human populations to mosquitoes in the Madang area, Papua New Guinea. Parasitology 96 (02), 251-263.
24. Peiris, J.S. et al. (1988) Monoclonal and polyclonal antibodies both block and enhance transmission of human Plasmodium vivax malaria. Am J Trop Med Hyg 39.
25. Premawansa, S. et al. (1994) Plasmodium falciparum malaria transmission-blocking immunity under conditions of low endemicity as in Sri Lanka. Parasite Immunol 16 (1), 35-42.
26. Ranawaka, M.B. et al. (1988) Boosting of transmission-blocking immunity during natural Plasmodium vivax infections in humans depends upon frequent reinfection. Infection and Immunity 56 (7), 1820-1824.
27. Mendis, K.N. et al. (1987) Malaria transmission-blocking immunity induced by natural infections of Plasmodium vivax in humans. Infection and Immunity 55 (2), 369-372.
28. Healer, J. et al. (1999) Transmission-blocking immunity to Plasmodium falciparum in malaria-immune individuals is associated with antibodies to the gamete surface protein Pfs230. Parasitology 119 (05), 425-433.
29. Mulder, B. et al. (1994) Malaria transmission-blocking activity in experimental infections of Anopheles gambiae from naturally infected Plasmodium falciparum gametocyte carriers. Trans. R. Soc. Trop. Med. Hyg. 88 (1), 121-125.
30. Naotunne, T.d.S. et al. (1990) Plasmodium cynomolgi: serum-mediated blocking and enhancement of infectivity to mosquitoes during infections in the natural host, Macaca sinica. Experimental parasitology 71 (3), 305-313.

678 31. de Arruda-Mayr, M. et al. (1979) Enhancement of a Simian Malarial Infection (Plasmodium
679 Cynomolgi) in Mosquitoes Fed on Rhesus (Macaca Mulatta) Previously Infected with an Unrelated
680 Malaria (Plasmodium Knowlesi)*. The American Journal of Tropical Medicine and Hygiene 28 (4),
681 627-633.
682 32. van der Kolk, M. et al. (2006) Reduction and enhancement of *Plasmodium falciparum*
683 transmission by endemic human sera. Int J Parasitol 36 (10–11), 1091-1095.
684 33. Carter, R. and Mendis, K. (1992) Transmission immunity in malaria: reflections on the underlying
685 immune mechanisms during natural infections and following artificial immunization. Mem Inst
686 Oswaldo Cruz 87, 169-173.
687 34. The malERA Consultative Group on Vaccines (2011) A Research Agenda for Malaria Eradication:
688 Vaccines. PLoS Med 8 (1), e1000398.
689 35. Ouédraogo, A.L. et al. (2018) Modeling the impact of Plasmodium falciparum sexual stage
690 immunity on the composition and dynamics of the human infectious reservoir for malaria in natural
691 settings. PLoS Path 14 (5), e1007034.
692 36. Stone, W.J.R. et al. (2018) Unravelling the immune signature of Plasmodium falciparum
693 transmission-reducing immunity. Nature Communications 9 (1), 558.
694 37. ClinicalTrials.gov [Internet]: National Library of Medicine (US), Safety and Immunogenicity of
695 Pfs25M-EPA/AS01 and Pfs230D1M-EPA/AS01 Vaccines, Transmission Blocking Vaccines Against
696 Plasmodium Falciparum, at Full and Fractional Dosing in Adults in Mali, Identifier: NCT02942277,
697 Bethesda (MD), Published: October 24, 2016, Accessed: August 22, 2018,
698 https://clinicaltrials.gov/ct2/show/NCT02942277
699 38. Saeed, M. et al. (2008) *Plasmodium falciparum* Antigens on the Surface of the Gametocyte-
700 Infected Erythrocyte. PLoS ONE 3 (5), e2280.
701 39. Dinko, B. et al. (2016) Antibody responses to surface antigens of Plasmodium falciparum
702 gametocyte-infected erythrocytes and their relation to gametocytaemia. Parasite Immunol 38 (6),
703 352-364.
704 40. Carter, R. et al. (1979) Plasmodium gallinaceum: Transmission-blocking immunity in chickens: II.
705 The effect of antigamete antibodies in vitro and in vivo and their elaboration during infection.
706 Experimental Parasitology 47 (2), 194-208.
707 41. Delves, M.J. et al. (2012) A high-throughput assay for the identification of malarial transmission-
708 blocking drugs and vaccines. Int J Parasitol 42 (11), 999-1006.
709 42. Ouédraogo, A.L. et al. (2013) A protocol for membrane feeding assays to determine the
710 infectiousness of *P. falciparum* naturally infected individuals to *Anopheles gambiae*. MWJ 4 (16).
711 43. Ponnudurai, T. et al. (1989) Infectivity of cultured Plasmodium falciparum gametocytes to
712 mosquitoes. Parasitology 98.
713 44. Ponnudurai, T. et al. (1982) Cultivation of fertile *Plasmodium falciparum* gametocytes in semi-
714 automated systems. 1. Static cultures. Trans. R. Soc. Trop. Med. Hyg. 76 (6), 812-818.
715 45. Bousema, T. et al. (2011) Human immune responses that reduce the transmission of Plasmodium
716 falciparum in African populations. Int J Parasitol 41 (3-4), 293-300.
717 46. Bousema, T. et al. (2012) Mosquito Feeding Assays to Determine the Infectiousness of Naturally
718 Infected *Plasmodium falciparum* Gametocyte Carriers. PLoS ONE 7 (8), e42821.
719 47. Roeffen, W. et al. (1994) Transmission blocking immunity as observed in a feeder system and
720 serological reactivity to Pfs 48/45 and Pfs230 in field sera. Mem Inst Oswaldo Cruz 89, 13-15.
721 48. van der Kolk, M. et al. (2005) Evaluation of the standard membrane feeding assay (SMFA) for the
722 determination of malaria transmission-reducing activity using empirical data. Parasitology 130 (01),
723 13-22.
724 49. Bolscher, J.M. et al. (2015) A combination of new screening assays for prioritization of
725 transmission-blocking antimalarials reveals distinct dynamics of marketed and experimental drugs. J
726 Antimicrob Chemother 70 (5), 1357-66.
727 50. Gwadz, R. (1976) Successful immunization against the sexual stages of Plasmodium gallinaceum.
728 Science 193 (4258), 1150-1151.

51. Carter, R. et al. (1979) Plasmodium gallinaceum: Transmission-blocking immunity in chickens: I. Comparative immunogenicity of gametocyte- and gamete-containing preparations. Experimental Parasitology 47 (2), 185-193.
52. Mendis, K.N. and Targett, G.A.T. (1981) Immunization to produce a transmission-blocking immunity in Plasmodium yoelii malaria infections. Trans. R. Soc. Trop. Med. Hyg. 75 (1), 158-159.
53. Gwadz, R.W. and Koontz, L.C. (1984) Plasmodium knowlesi: persistence of transmission blocking immunity in monkeys immunized with gamete antigens. Infection and Immunity 44 (1), 137-140.
54. Gwadz, R.W. and Green, I. (1978) Malaria immunization in Rhesus monkeys. A vaccine effective against both the sexual and asexual stages of Plasmodium knowlesi. The Journal of experimental medicine 148 (5), 1311-1323.
55. Graves, P.M. et al. (1988) Antibodies to Plasmodium falciparum gamete surface antigens in Papua New Guinea sera. Parasite Immunol 10 (2), 209-218.
56. Drakeley, C.J. et al. (1998) Transmission-blocking effects of sera from malaria-exposed individuals on Plasmodium falciparum isolates from gametocyte carriers. Parasitology 116 (05), 417-423.
57. Reuling, I.J. et al. (2018) A randomized feasibility trial comparing four antimalarial drug regimens to induce Plasmodium falciparum gametocytemia in the controlled human malaria infection model. eLife 7, e31549.
58. Collins, K.A. et al. (2018) A controlled human malaria infection model enabling evaluation of transmission-blocking interventions. The Journal of Clinical Investigation 128 (4), 1551-1562.
59. Bousema, J.T. et al. (2006) Rapid onset of transmission-reducing antibodies in javanese migrants exposed to malaria in papua, indonesia. The American journal of tropical medicine and hygiene 74 (3), 425-431.
60. Bousema, J.T. et al. (2007) A longitudinal study of immune responses to Plasmodium falciparum sexual stage antigens in Tanzanian adults. Parasite Immunol 29 (6), 309-317.
61. Bousema, T. et al. (2010) The Dynamics of Naturally Acquired Immune Responses to *Plasmodium falciparum* Sexual Stage Antigens Pfs230 & Pfs48/45 in a Low Endemic Area in Tanzania. PLoS ONE 5 (11), e14114.
62. Ponnudurai, T. et al. (1987) Transmission blockade of Plasmodium falciparum: its variability with gametocyte numbers and concentration of antibody. Trans. R. Soc. Trop. Med. Hyg. 81 (3), 491-493.
63. Miura, K. et al. (2016) An inter-laboratory comparison of standard membrane-feeding assays for evaluation of malaria transmission-blocking vaccines. Malaria Journal 15 (1), 463.
64. Canepa, G.E. et al., Antibody targeting of a specific region of Pfs47 blocks Plasmodium falciparum malaria transmission, NPJ Vaccines, 2018, p. 26.
65. Guevara Patiño, J.A. et al. (1997) Antibodies that Inhibit Malaria Merozoite Surface Protein–1 Processing and Erythrocyte Invasion Are Blocked by Naturally Acquired Human Antibodies. The Journal of Experimental Medicine 186 (10), 1689-1699.
66. Da, D.F. et al. (2015) Experimental study of the relationship between Plasmodium gametocyte density and infection success in mosquitoes; implications for the evaluation of malaria transmission-reducing interventions. Experimental Parasitology 149, 74-83.
67. Bradley, J. et al. (2018) Predicting the likelihood and intensity of mosquito infection from sex specific Plasmodium falciparum gametocyte density. eLife 7, e34463.
68. Gonçalves, B.P. (2017) The human infectious reservoir for Plasmodium falciparum malaria in areas of differing transmission intensity. Under review.
69. Blagborough, A.M. et al. (2013) Transmission-blocking interventions eliminate malaria from laboratory populations. Nature Communications 4, 1812.
70. Churcher, T.S. et al. (2015) Human-to-mosquito transmission efficiency increases as malaria is controlled. Nat Commun 6.
71. Rono, M.K. et al. (2018) Adaptation of Plasmodium falciparum to its transmission environment. Nature Ecology & Evolution 2 (2), 377-387.
72. Theisen, M. et al. (2017) Towards clinical development of a Pfs48/45-based transmission blocking malaria vaccine. Expert Review of Vaccines 16 (4), 329-336.

780 73. Churcher, T.S. et al. (2013) Predicting mosquito infection from Plasmodium falciparum
781 gametocyte density and estimating the reservoir of infection. Elife 2, e00626.
782 74. Da, D.F. et al. (2015) Experimental study of the relationship between Plasmodium gametocyte
783 density and infection success in mosquitoes; implications for the evaluation of malaria transmission-
784 reducing interventions. Exp Parasitol 149, 74-83.
785 75. Chansamut, N. et al. (2012) Correlation of Pfg377 ortholog gene expression of Plasmodium vivax
786 and mosquito infection. Tropical Medicine & International Health 17 (4), 414-422.
787 76. Robert, V. et al. (2003) Sex ratio of Plasmodium falciparum gametocytes in inhabitants of Dielmo,
788 Senegal. Parasitology 127 (Pt 1), 1-8.
789 77. Stone, W.J.R. et al. (2016) Naturally acquired immunity to sexual stage P. falciparum parasites.
790 Parasitology 143 (2), 187-198.
791 78. Naotunne, T.S. et al. (1991) Cytokines kill malaria parasites during infection crisis: extracellular
792 complementary factors are essential. The Journal of Experimental Medicine 173 (3), 523-529.
793 79. Naotunne, T.S. et al. (1993) Cytokine-mediated inactivation of malarial gametocytes is
794 dependent on the presence of white blood cells and involves reactive nitrogen intermediates.
795 Immunology 78 (4), 555-562.
796 80. Healer, J. et al. (1999) Phagocytosis Does Not Play a Major Role in Naturally Acquired
797 Transmission-Blocking Immunity to Plasmodium falciparum Malaria. Infection and Immunity 67 (5),
798 2334-2339.
799 81. Sinden, R.E. and Smalley, M.E. (1976) Gametocytes of Plasmodium falciparum: phagocytosis by
800 leucocytes in vivo and in vitro. Trans. R. Soc. Trop. Med. Hyg. 70 (4), 344-345.
801 82. Sutherland, C.J. (2009) Surface antigens of Plasmodium falciparum gametocytes—A new class of
802 transmission-blocking vaccine targets? Mol Biochem Parasitol 166 (2), 93-98.
803 83. Florens, L. et al. (2002) A proteomic view of the Plasmodium falciparum life cycle. Nature 419
804 (6906), 520-526.
805 84. Rener, J. et al. (1983) Target antigens of transmission-blocking immunity on gametes of
806 plasmodium falciparum. The Journal of experimental medicine 158 (3), 976-981.
807 85. Premawansa, S. et al. (1990) Target antigens of transmission blocking immunity of Plasmodium
808 vivax malaria. Characterization and polymorphism in natural parasite isolates. The Journal of
809 Immunology 144 (11), 4376-4383.
810 86. Vermeulen, A.N. et al. (1985) Sequential expression of antigens on sexual stages of *Plasmodium*
811 *falciparum* accessible to transmission-blocking antibodies in the mosquito. The Journal of
812 Experimental Medicine 162 (5), 1460-1476.
813 87. Quakyi, I.A. et al. (1987) The 230-kDa gamete surface protein of Plasmodium falciparum is also a
814 target for transmission-blocking antibodies. The Journal of Immunology 139 (12), 4213-4217.
815 88. Roeffen, W. et al. (1995) Transmission blockade of Plasmodium falciparum malaria by anti-
816 Pfs230-specific antibodies is isotype dependent. Infection and Immunity 63 (2), 467-71.
817 89. Healer, J. et al. (1997) Complement-mediated lysis of Plasmodium falciparum gametes by
818 malaria-immune human sera is associated with antibodies to the gamete surface antigen Pfs230.
819 Infection and Immunity 65 (8), 3017-23.
820 90. Silvestrini, F. et al. (2010) Protein Export Marks the Early Phase of Gametocytogenesis of the
821 Human Malaria Parasite Plasmodium falciparum. Molecular & Cellular Proteomics 9 (7), 1437-1448.
822 91. Meerstein-Kessel, L. et al. (2018) Probabilistic data integration identifies reliable gametocyte-
823 specific proteins and transcripts in malaria parasites. Sci Rep 8 (1), 410.
824 92. van Dijk, M.R. et al. (2001) A Central Role for P48/45 in Malaria Parasite Male Gamete Fertility.
825 Cell 104 (1), 153-164.
826 93. Eksi, S. et al. (2006) Malaria transmission-blocking antigen, Pfs230, mediates human red blood
827 cell binding to exflagellating male parasites and oocyst production. Mol Microbiol 61 (4), 991-998.
828 94. Eksi, S. et al. (2002) Targeting and sequestration of truncated Pfs230 in an intraerythrocytic
829 compartment during Plasmodium falciparum gametocytogenesis. Mol Microbiol 44 (6), 1507-1516.

95. Drakeley, C.J. et al. (2006) Transmission-reducing immunity is inversely related to age in Plasmodium falciparum gametocyte carriers. Parasite Immunol 28 (5), 185-190.
96. Miura, K. et al. (2016) Transmission-blocking activity is determined by transmission-reducing activity and number of control oocysts in Plasmodium falciparum standard membrane-feeding assay. Vaccine. 34.
97. Muirhead-Thomson, R.C. (1954) Factors determining the true reservoir of infection of *Plasmodium falciparum* and *Wuchereria bancrofti* in a West African village. Trans. R. Soc. Trop. Med. Hyg. 48 (3), 208-225.
98. Gneme, A. et al. (2013) Equivalent susceptibility of *Anopheles gambiae* M and S molecular forms and *Anopheles arabiensis* to *Plasmodium falciparum* infection in Burkina Faso. Malaria Journal 12 (1), 204.
99. Johnston, G.L. et al. (2013) Malaria's missing number: calculating the human component of R0 by a within-host mechanistic model of Plasmodium falciparum infection and transmission. PLoS Comp Biol 9 (4), e1003025.
100. Garber, K. (2014) Bispecific antibodies rise again. Nature Reviews Drug Discovery 13, 799.