ADVANCES ON THE GAMETOCYTE BIOLOGY, HOST IMMUNITY AND VECTOR STAGES TO INTERRUPT THE TRANSMISSION OF MALARIA

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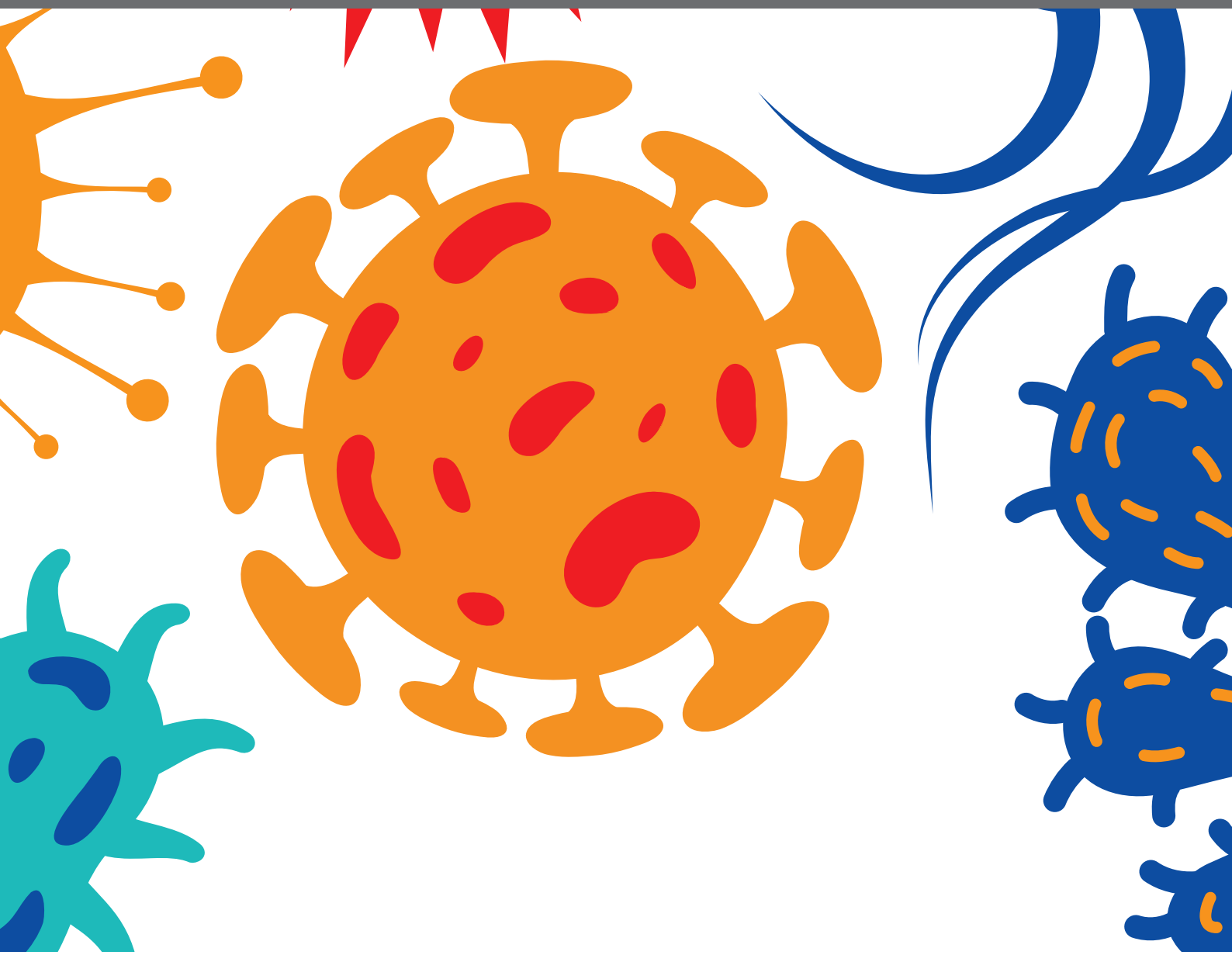

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ADVANCES ON THE GAMETOCYTE BIOLOGY, HOST IMMUNITY AND VECTOR STAGES TO INTERRUPT THE TRANSMISSION OF MALARIA

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Table of Contents

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EDITORIAL
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Editorial: Advances on the Gametocyte Biology, Host Immunity and Vector Stages to Interrupt the Transmission of Malaria

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Editorial on the Research Topic

Advances on the Gametocyte Biology, Host Immunity and Vector Stages to interrupt the Transmission of Malaria

Stringent malaria control efforts have reduced the global malaria burden in the past decade. However, the decrease in case numbers has plateaued and increasing drug and insecticide resistance has made the development of new interventions a top priority. Further, this decrease has been unravelled by the on-going COVID-19 pandemic and the associated disruptions in access to global malaria interventions (WHO, 2021). In 2020, there were an estimated 241 million cases and 627,000 malaria deaths, an increase of over 14 million cases and 69,000 deaths compared to 2019 (WHO, 2021).

While much of the previous malaria control efforts have been focused on the pre-erythrocytic and blood-stages of malaria, specifically inhibiting the transmission of parasites from the human host to the mosquito vector is now recognized as a key step for elimination (MVF, 2013). Targeting the transmission stages of the parasite will confer protection on a population level by inhibiting the onward transmission from an infected individual to the next.

The transmission stages of malaria are complex and offer multiple opportunities for intervention. Various environmental and genetic signals trigger the switch from asexually replicating forms within the human host to sexual gametocytes. In the human host, gametocytes, still residing within red blood cells, sequester and develop in the extra-vasculature of hematopoietic niches, such as the bone marrow and spleen. At maturation, male and female gametocytes are released back into the peripheral circulation and can be taken up by mosquitoes during blood feeds to complete transmission from human to mosquito. In the mosquito midgut, male and female gametes emerge to undergo sexual replication through fertilisation, resulting in the formation of zygotes. These develop into actively moving ookinetes that move through the midgut epithelium to develop

into oocysts in the surface of the midgut. Upon maturation, oocytes burst to release sporozoites into the body cavity which then migrate to the salivary glands to start a new cycle of infection upon the next blood meal. Antigens that are expressed during this stage of the parasite life cycle, specifically on the surface of gametocytes and gametes, represent attractive targets of antibodies, which can contribute to the development of effective vaccines (Stone et al., 2018).

The reviews and original research articles in this Research Topic feature the recent progress in this understudied area and highlight important knowledge gaps that need addressing, in order to develop new transmission blocking interventions. Articles in the first section describe the natural acquisition of antibodies to antigen targets on the surface of gametocytes in endemic populations. Muthui et al. demonstrated the acquisition of human antibodies to gametocyte surface antigens, including the lead vaccine candidate Pfs230 using two clinical cohorts from an endemic region in Kenya. Further, they identified a panel of new gametocyte antigens with the potential to be further evaluated as transmission-blocking vaccine candidates. Broni et al. examined the levels and avidity of naturally induced antibody responses against vaccine candidates Pfs48/45 and Pfs230, from the end of a peak malaria season to the start of the subsequent season. O'Flaherty et al. examined associations between antibody levels to Pfs230 and Pfs48/45 and concurrent gametocytemia across different transmission settings using human samples from a multi-national clinical study. They showed that Pfs230 and Pfs48/45 are targets of natural immunity that are associated with gametocyte densities across multiple endemic settings.

Takashima et al. reviewed the progress and challenges in identifying new and effective transmission-blocking vaccine candidates. To accelerate the process of antigen discovery for transmission-blocking vaccines, they proposed the use of genome-wide approaches to identify key target antigens. These will be subsequently expressed using a recombinant system that produces correctly folded proteins to evaluate for transmission-blocking activity. Another review article by Yu et al. highlights the potential of biological control strategies that directly kill the mosquito vector as an effort to interrupt malaria transmission. They further discuss the ongoing infectivity of patients after clearance of blood stage parasitemia as gametocytes are not effectively targeted by the current gold-standard ACT treatment. The only currently licensed drug that kills gametocytes is Primaquine, but wide usage is prohibited by safety issues in G6PD deficient patients. Atovaquone/Proguanil have been shown to have transmission blocking activity by targeting mosquito stages of the parasites, however a short drug half-life is problematic. These are areas where further research is needed and offers potential for further targeted intervention.

The next section of articles comprises original research and review articles on the commitment to sexual development and transmission dynamics. *Plasmodium* parasites are able to adjust commitment to gametocyte conversion and investment in transmission in areas of changing environments. Importantly, investment into gametocytes for both *P. falciparum* and *P. vivax* has been shown to be higher in areas of lower transmission (Rono et al., 2018, Koepfli et al., 2021), whereas seasonal changes appear to have an opposite effect with higher transmission in the wet season (reviewed in Oduma and Koepfli). This adaptation to changes in transmission, and in particular differences between Pf and Pv transmission biology needs to be addressed when designing novel transmission blocking interventions. Methodologies to assess gametocyte conversion rates, transmission potential and gametocyte infectivity are summarized in Oduma and Koepfli l., while Ford et al. presented novel biomarkers of gametocyte presence. Thommen et al. addressed the important question of whether drug induced stress responses can lead to an increased commitment to sexual development. Usui and Willimson. provide a comprehensive review and discuss transmission dynamics and identify knowledge gaps. Timinao et al. investigated the infectivity of different human to malaria reservoirs to estimate their contribution to the transmission of malaria. They recruited symptomatic patients from an endemic region in Papua New Guinea and showed through direct membrane feeding assays that the majority of gametocyte-positive individuals were able to infect *Anopheles* mosquitoes. Further, they showed that acute *P. vivax* infections led to more frequent mosquito infections as their gametocytes developed much quicker than those of *P. falciparum*.

Together, the collection of articles in this Research Topic emphasizes the importance of further research into transmission-blocking approaches that is key for achieving sustained malaria elimination.

AUTHOR CONTRIBUTIONS

J-AC and LR contributed equally to writing the manuscript. PN edited sections of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

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ORIGINAL RESEARCH
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Characterization of Naturally Acquired Immunity to a Panel of Antigens Expressed in Mature *P. falciparum* Gametocytes

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Introduction: Naturally acquired immune responses against antigens expressed on the surface of mature gametocytes develop in individuals living in malaria-endemic areas. Evidence suggests that such anti-gametocyte immunity can block the development of the parasite in the mosquito, thus playing a role in interrupting transmission. A better comprehension of naturally acquired immunity to these gametocyte antigens can aid the development of transmission-blocking vaccines and improve our understanding of the human infectious reservoir.

Methods: Antigens expressed on the surface of mature gametocytes that had not previously been widely studied for evidence of naturally acquired immunity were identified for protein expression alongside Pfs230-C using either the mammalian HEK293E or the wheat germ cell-free expression systems. Where there was sequence variation in the candidate antigens (3D7 *vs* a clinical isolate PfKE04), both variants were expressed. ELISA was used to assess antibody responses against these antigens, as well as against crude stage V gametocyte extract (GE) and AMA1 using archived plasma samples from individuals recruited to participate in malaria cohort studies. We analyzed antibody levels (estimated from optical density units using a standardized ELISA) and seroprevalence (defined as antibody levels greater than three standard deviations above the mean levels of a pool of malaria naïve sera). We described the dynamics of antibody responses to these antigens by identifying factors predictive of antibody levels using linear regression models.

Results: Of the 25 antigens selected, seven antigens were produced successfully as recombinant proteins, with one variant antigen, giving a total of eight proteins for evaluation. Antibodies to the candidate antigens were detectable in the study population (N = 216), with seroprevalence ranging from 37.0% (95% CI: 30.6%, 43.9%) for PSOP1 to 77.8% (95% CI: 71.6%, 83.1%) for G377 (3D7 variant). Responses to AMA1 and GE were more prevalent than those to the gametocyte proteins at 87.9% (95% CI: 82.8%, 91.9%) and 88.3% (95% CI: 83.1%, 92.4%), respectively. Additionally, both antibody levels and breadth of antibody responses were associated with age and concurrent parasitaemia.

Conclusion: Age and concurrent parasitaemia remain important determinants of naturally acquired immunity to gametocyte antigens. Furthermore, we identify novel candidates for transmission-blocking activity evaluation.

Keywords: *Plasmodium falciparum*, naturally acquired immunity, mature gametocytes, seroepidemiology, malaria transmission

1 INTRODUCTION

Early investigations into immune responses to gametocyte antigens demonstrated that they are readily detectable in the sera of malaria exposed individuals and can sometimes develop rapidly after primary infection (Mendis et al., 1987; Graves et al., 1988; Ong et al., 1990; Bousema et al., 2006). The destruction of circulating gametocytes within the human host before transmission to mosquitoes results in gametocyte proteins being presented to the vertebrate immune system, thus stimulating an immune response (Pradel, 2007; Stone et al., 2016). The naturally acquired anti-gametocyte immune response is predominantly humoral, and work has shown that it can impact parasite development within the mosquito (Oueadraogo et al., 2018; Stone et al., 2018). Therefore, there is the potential for these immune responses to subsequently influence transmission with impact on the infectious reservoir (Stone et al., 2018). Data on naturally acquired transmission-blocking immunity may therefore inform the development of transmission-blocking vaccines (Bousema et al., 2010).

In seroepidemiological studies, serological status is defined by the presence or levels of antibodies to key parasite antigens and is used as a marker of individual or population-level parasite exposure (Polley et al., 2004; Drakeley et al., 2005; Wong et al., 2014; Kangoye et al., 2016; Idris et al., 2017). Such studies can be used to identify factors associated with the carriage of antibodies to *Plasmodium falciparum* antigens. Some of the key indicators of parasite exposure include age, location of residence and asymptomatic parasitaemia, which are commonly assessed for associations with immune responses to parasite antigens. From the seroepidemiological studies carried out so far on *P. falciparum* gametocyte antigens, based primarily on Pfs230-C and Pfs48/45, there exist discrepancies in the associations observed with age, transmission intensity and transmission season (Muthui et al., 2019a). Further work is therefore required to clarify these associations.

Several parameters influence gametocyte carriage, for example, host genetics, in particular the haemoglobinopathies that confer protection against severe malaria (Williams et al., 2005; Taylor et al., 2012; Ndungu et al., 2015). Furthermore, gametocyte carriage is also likely to impact naturally acquired immune responses to gametocyte antigens. Based on this premise, we identified a set of largely uncharacterized antigens for immunoprofiling in relation to well-studied serological markers of parasite exposure as well as risk factors for gametocyte carriage. Through this analysis, we highlight important factors that modulate the anti-gametocyte antibody response (age and concurrent parasitaemia), highlight potential markers of parasite exposure as well as new candidates that can be evaluated for transmission-blocking activity.

2 METHODS

2.1 Study Design, Setting and Data Collection

Samples and epidemiological data from two cohorts were used, being the Kilifi malaria longitudinal cohort [KMLC study (Muthui et al., 2019b)] and the assessment of the infectious reservoir of malaria [AFIRM study (Gonçalves et al., 2017)]. The KMLC cohort comprised three sub-cohorts of children followed up longitudinally and sampled at cross-sectional surveys to assess asymptomatic *P. falciparum* infections. The AFIRM cohort was a cross-section sampling carried out in the wet and dry seasons and comprised children and adults. A breakdown of the cohorts is provided in **Table 1**.

2.1.1 Kilifi Malaria Longitudinal Cohort (KMLC)

The KMLC cohort study design and sampling protocol and the subset of samples used in this study have previously been described (Mwangi et al., 2005; Bejon et al., 2006; Muthui et al., 2019b; Omondi et al., 2021). Briefly, a subset of samples was selected for the analysis, including all gametocyte positive children sampled over the 1998 – 2016 follow-up period. This gave a total of 364 samples available for analysis. Controls were selected and matched on age, sex and cohort. One set of controls was asexual parasite positive but gametocyte negative by microscopy, and the other

TABLE 1 | Summary of the cohorts included in the immunoprofiling.

Cohort	Location (s)	Study Design	Period of Sample Collection	Population Sampled	Sample Size
KMLC	Ngerenya and Junju	Cross-sectional surveys	1998 - 2016	Children	272
AFIRM	Junju	Cross-sectional (seasonally spaced)	January 2014 - February 2015	Children and Adults	216

negative for both asexual parasites and gametocytes. Not all samples were available for the eventual analysis. The final sample set consisted of 66 of the gametocyte-positive, 72 of the asexual parasite positive, and 134 of the parasite negative samples, giving 272 samples. A figure providing the sample selection process is provided in **Supplementary Figure 1**. Owing to the decline in transmission in the Ngerenya sub-cohort from 2001, the sub-cohort was divided into Ngerenya-early (1998 – 2001), a period of moderate transmission and Ngerenya-late (2002 – 2016), a period of low transmission. Similar to Ngerenya-early, the Junju sub-cohort is located within a region experiencing moderate malaria transmission.

2.1.2 Assessment of the Infectious Reservoir of Malaria (AFIRM)

The AFIRM study was carried out in Junju location, Kilifi, to describe the infectious reservoir in an area of moderate malaria transmission intensity. Details of this cohort have previously been published (Gonçalves et al., 2017). The study design was cross-sectional, with one survey carried out during the rainy season and one in the dry season. Though increased transmission occurs following the onset of the rainy season, parasite transmission persists all year round along the Kenyan coast, and the timing of the rains and consequent increase in malaria transmission is irregular (Mbogo et al., 1995; Mogeni et al., 2016). There is no standard definition of the start and the end of the malaria transmission season. In our study, the start and end of the dry and wet seasons were defined using monthly rainfall data collected between 2013 – 2015. Both children and adults were recruited into the study, with different participants recruited at each cross-sectional survey. Recruitment was carried out between January 2014 and April 2014 for the dry season, with additional participants recruited between January 2015 and February 2015 and for the rainy season between May and December. Participants were recruited regardless of parasite status, with the key inclusion criteria being informed consent, age greater than two years, and willingness to provide a single 5 ml venous blood sample. The main exclusion criteria were acute disease or severe chronic conditions. At recruitment, rapid diagnostic tests were performed using Carestart RDT®, and any individuals presenting with parasites, by microscopy, were given a full course of anti-malarial treatment. A subset of 216 samples (72 from adults, 72 from children over five years and 72 from children under five years) was randomly selected from the main AFIRM dataset for analysis. A figure providing the sample selection process is provided in **Supplementary Figure 1**.

2.1.3 Ethics Approval

The cohort studies described above were approved by the Kenya Medical Research Institute Ethics Review Committee (reference numbers KEMRI/SERU/SSC2574 - AFIRM cohort, and KEMRI/SERU/CGMRC//3149 and KEMRI/SERU/SSC1131 - KMLC cohort). The research was conducted in line with the principles of the Declaration of Helsinki, which included consenting the participants in their local language before any procedure, and obtaining written consent from the participants, or in the case of children, from their parents or guardians.

2.1.4 Parasite Detection

For both the KMLC and AFIRM cohorts, data on patent parasitaemia was available. The microscopy protocol has previously been described (Mwangi et al., 2005; Muthui et al., 2019b). Briefly, blood films were prepared for the participants, fixed with 100% methanol, and subsequently stained for 45 minutes with 3% Giemsa. Enumeration of parasites on thick films was estimated relative to a count of 200 white blood cells (WBCs), while a count of 500 red blood cells (RBCs) was used for thin films. Final parasitaemia was then determined against the actual blood count or by assuming a count of either 8000 WBCs per microlitre or 5 x 10^6 RBCs per microlitre. For the AFIRM cohort, the parasite density was calculated based on an estimate of 8000 WBCs per microlitre. Gametocyte detection was done alongside the detection of asexual parasites. All microscopy readings were carried out independently by two microscopists, with any discrepancies resolved by a third microscopist. In addition to microscopy, parasites were also detected by molecular methods for the AFIRM cohort. Detection of all parasites was done by *18S* rRNA quantitative nucleic acid sequence-based amplification (QT-NASBA) and *18S* qPCR, while specific detection of female gametocytes was carried out by *Pfs25* mRNA QT-NASBA (Schneider et al., 2004; Pett et al., 2016).

2.2 Identification of Antigens for Study

Candidate antigens for the study were identified from a published dataset of the gametocyte proteome (Lasonder et al., 2016). From an initial list of 2,241 proteins, we shortlisted 24 proteins for further analysis. These proteins were shortlisted based on features suggestive of surface localization (signal peptides, transmembrane domains and glycosylphosphatidylinositol anchors). An additional antigen with potential association with naturally acquired transmission reducing immunity was identified from a conference abstract (Stone et al., 2015) to give a total of 25 proteins (**Supplementary Table 1**). At the time of the search, the candidate antigens were predominantly uncharacterized as targets of naturally acquired immunity to mature gametocyte antigens. Sequence variation between the reference lab strain *P. falciparum* 3D7 (genome version 3.0) and a fully sequenced Kilifi clinical isolate – PfKE04 – was also analyzed by pairwise alignment using Geneious bioinformatics software (version

11.1.2). Where variation was identified [either an insertion/deletion or non-synonymous single nucleotide polymorphism (SNP)], both variants were prioritized for construct design.

Protein production was attempted in the wheat germ cell-free expression system (WGCFS, WEPRO7240H, Cell-Free Sciences) and, where unsuccessful in the WGCFS, in the mammalian HEK293E system. Of the 25 antigens targeted for recombinant protein production, seven antigens (including one variant antigen) were successfully expressed in sufficient quantities for this analysis. These were Pfs230-C (*PF3D7_0209000*), *PF3D7_0208800*, MDV1 (*PF3D7_1216500*), G377 (*PF3D7_1250100*, 3D7 and PfKE04 variants expressed), *PF3D7_1314500*, *PF3D7_0303900* and PSOP1 (*PF3D7_0721700*)) giving a total of 7 candidate antigens (8 proteins) for immunoprofiling analysis. Notably, owing to the size of G377 protein that would be a challenge for protein production, a domain spanning amino acids 666-1146 termed 'domain B' was produced for analysis. This domain was identified from work by Alano et al. (1995), who were able to produce protein from this domain successfully (Alano et al., 1995). An extensive description of the protein identification and the recombinant protein production process is provided in the **Supplementary Methods**, and results from the protein production are provided in **Supplementary Figures 2–5**. Additionally, we evaluated antibody responses to apical membrane antigen-1 protein (AMA1, *PF3D7_1133400*), a highly immunogenic asexual stage antigen (Drakeley et al., 2005; Jones et al., 2015; Idris et al., 2017) widely studied in the context of seroprevalence to place our results in context, and to a crude stage V gametocyte extract (GE).

2.3 Enzyme-Linked Immunosorbent Assay (ELISA)

The measurement of antibody responses to the candidate antigens, AMA1 and GE, was carried out using a previously described three-day ELISA protocol (Murungi et al., 2019; Omondi et al., 2021) with a few modifications. Additionally, to determine the optimal antigen coating concentration and serum dilution to use for the assay, checkerboard ELISAs were first carried out (**Supplementary Figures 6, 7**). On day one of the assay, 100 µL of the purified recombinant protein was prepared in coating buffer (at a concentration of 1 µg/ml for the gametocyte antigens, 0.5 µg/ml for AMA1 and 1 in 250 dilution for GE) and coated onto a 96-well plate (Immulon 4 HBX, ThermoFisher Scientific). The plate was then incubated at 4°C overnight, and on the following day, the plate was washed four times with phosphate-buffered saline containing 0.05% tween-20 (Pierce™ 20X PBS Tween™ 20 Buffer, ThermoFisher Scientific). The plate was then blocked with 200 µL of blocking buffer for five hours at room temperature and subsequently washed three times with PBS/T. One hundred microlitres of test sera were then added at dilutions of 1 in 200 for the gametocyte antigens (or 1 in 100 for lowly reactive antigens - i.e., if average optical densities were below 0.15); 1 in 1000 (or 1 in 2000 for adult sera tested) for AMA1, and 1 in 500 for GE). A standardized ELISA format was used where defined concentrations of malaria immunoglobulin (MIG) were serially diluted to generate a standard curve against which antibody units were extrapolated.

The MIG was prepared from purified immunoglobulin G (98% IgG) obtained from a pool of 834 Malawian adult sera (Taylor et al., 1992). For positive controls, a characterized pool of hyper-immune sera (PHIS) was obtained from a random selection of adult residents of Junju location, Kilifi (Osier et al., 2014; Murungi et al., 2016), and a pool of sera from gametocyte positive individuals from the AFIRM cohort with high gametocytaemia were also included. Malaria naive sera from European adults (non-immune sera, NIS) were used as negative controls at the same dilution as the test serum for each respective antigen. The NIS were tested against the measles antigen to verify that they were indeed reactive (**Supplementary Figure 8**). On day three, the plate was washed three times before 100 µL of secondary antibody (polyclonal rabbit anti-human IgG-HRP, Dako, UK) was added (1: 5,000 dilution) and the plate incubated at room temperature for three hours. The plate was washed four times before adding 100 µL of o-phenylenediamine dihydrochloride (OPD) substrate (Sigma, UK). Finally, the plate was incubated at room temperature for 15 minutes to allow colour development before the reaction was stopped using 25 µL of 2 M sulphuric acid (H_2SO_4). Each sample's optical density (OD) was then determined by reading the absorbance at 492 nM. All samples were run in duplicate, with replicate samples run on a separate plate. As all samples were run in duplicate in the ELISA, the mean OD and co-efficient of variation were calculated before analysis. Samples with a coefficient of variation higher than 20% were repeated.

2.4 Statistical Analysis

We summarised the demographic characteristics of participants for the two cohorts separately. We did this by sub-cohort (Ngerenya-early, Ngerenya-late and Junju) within the KMLC cohort and by season (wet and dry) within the AFIRM cohort. For continuous variables, we presented their means (standard deviation) or median (interquartile range, IQR) depending on the distribution. For categorical variables, we presented the percentages with comparisons carried out using the Chi-square test. We also summarised parasite density in the different cohorts by age group, sickle cell status, α–thalassaemia status, cohort and season. Comparisons between groups were performed using the Wilcoxon rank-sum test (where a categorical variable had only two groups) or Kruskal-Wallis test (for variables with more than two groups) with posthoc analysis done using the Dunn's test with Bonferroni correction.

Next, we estimated the seroprevalence. We note that the analysis of seroprevalence was limited to the AFIRM cohort only, where sampling cut across all age groups. Antibody responses (IgG) were measured to the candidate antigens that were successfully produced as recombinant proteins. Responses were also measured to two markers of parasite exposure: Apical membrane antigen 1 (AMA1) (Drakeley et al., 2005; Jones et al., 2015; Idris et al., 2017) and gametocyte extract (GE) (Omondi et al., 2021). A four-parameter hyperbolic standard curve was generated from the titrated MIG, and the antibody concentration of each sample was then extrapolated. A cut-off was defined as the mean antibody

concentration of the NIS plus three standard deviations (3SD) to determine seropositivity. Additionally, to compare the different methods of assigning seropositivity, we also calculated seropositivity using a mixed model with the distribution of OD values fitted as the sum of two Gaussian distributions (a narrow distribution of low responders and a broader distribution of high responders) (Bousema et al., 2010). We then estimated seropositivity from a cut-off defined as the mean OD of the low responders plus 3SD. These seroprevalences were compared across variant antigens and across sampling seasons by means of a Chi-square test. The Cochrane-Armitage test was used to test for a linear trend in seroprevalence when grouped by age. Additionally, we also evaluated the dependency of seropositivity to the different antigens using a correlation matrix, with correlation analyzed using Spearman's rank correlation.

Furthermore, we examined how determinants of parasite exposure such as age, parasite prevalence, season (AFIRM cohort), transmission intensity (KMLC cohort), and haemoglobinopathies relate to the magnitude of antibody response to the candidate antigens. These covariates were chosen owing to their associations with gametocytaemia or anti-gametocyte antibody responses (Boudin et al., 1991; Lawaly et al., 2010; Grange et al., 2015; Skinner et al., 2015; Amoah et al., 2018). Factors associated with the magnitude and breadth of antibody response to the gametocyte antigens were identified using linear regression models, and we fitted both univariable and multivariable models. Cluster-robust standard errors were calculated with clustering specified to occur by participant ID, to account for instances of repeated sampling in the KMLC cohort. For the AFIRM cohort, parasitaemia and gametocytaemia were assessed by both microscopy and the highly sensitive nucleic acid sequence-based amplification; thus, it was also possible to compare the effect of patent versus sub-patent parasitaemia and gametocytaemia in a sub-analysis.

In addition to assessing the factors associated with the magnitude of anti-gametocyte responses, we also assessed the parameters associated with the recognition of a greater number of gametocyte antigens (breadth of response). For this, we considered how the pre-specified covariates related to the breadth of antibody responses to the candidate antigens using linear regression models. All analyses were performed using R version 4.0.3 (R Core Team, 2020).

3 Results

3.1 Demographic Characteristics

3.1.1 Kilifi Malaria Longitudinal Cohort (KMLC) Study

Table 2 presents the demographic characteristics of the KMLC. In all sub-cohorts, observations were predominantly from children below five years of age. The median age across all sub-cohorts was 5.22 years (IQR 4.98 years), with the proportion under five years of age being: Ngerenya-early - 64%, Ngerenya-late - 60% and Junju - 52% ($p = 0.302$, Chi-square test). Parasite prevalence, detected by microscopy, was 78% in Ngerenya-early, 60.4% in Junju and 19% in Ngerenya-late for asexual parasites and 25% in Ngerenya-early, 33% in Junju and 8% in Ngerenya-late for gametocytes as detected by microscopy. Owing to the unavailability of a majority of samples (**Supplementary Figure 1**), it was impossible to undertake the initial matching of cases and controls, which is reflected in the variable parasite prevalence in the sub-cohorts.

TABLE 2 | Demographic characteristics of observations from a subset of the KMLC cohort study participants.

	Sub-cohort		
	Ngerenya		Junju
	Early	Late	
Number of observations (N)	50	126	96
Sex: number of females (%)	25 (50.0)	40 (31.7)	45 (46.9)
Number per age group (%)			
0 - 5 years	32 (64.0)	76 (60.3)	50 (52.1)
6 - 10 years	14 (28.0)	41 (32.5)	29 (30.2)
11-15 years	4 (8.0)	9 (7.1)	17 (17.7)
Temperature (°C), median (IQR)	NM*	36.9 (36.6 - 37.1)	36.5 (36.2 - 36.9)
Number of asexual parasite positive observations (%)	39 (78.0)	24 (19.0)	58 (60.4)
Number of gametocyte positive observations (%)	25 (50.0)	8 (6.3)	33 (34.4)
Number of observations with sickle genotype (%)			
AA	49 (98.0)	103 (82.4)	70 (72.9)
AS	1 (2.0)	22 (17.6)	26 (27.1)
Number of observations with α-thalassaemia genotype (%)			
Normal	18 (40.9)	39 (31.7)	31 (32.3)
Heterozygous	21 (47.7)	65 (52.8)	48 (50.0)
Homozygous	5 (11.4)	19 (15.4)	17 (17.7)
Missing data			
Sickle genotype	.	1	.
α-thalassaemia genotype	6	3	.

**NM, not measured. A dot (.) indicates that no data were missing for the particular variable, while a number indicates the number of participants for whom corresponding genotype data were not available.*

The prevalence of sickle cell trait (AS) varied among the sub-cohorts (p =0.001, Chi-square test) with higher prevalence in Junju (27%) and Ngerenya-late (17.6%) and lowest prevalence in Ngerenya-early (2%). The markedly low prevalence of sickle cell trait in Ngerenya-early likely results from an artefact of sample availability rather than reflecting an increase in the prevalence of sickle cell trait over time. In comparison, close to half the participants were heterozygous for α–thalassaemia (Ngerenya-early 47.7%, Ngerenya-late 52.8% and Junju 50%, $p = 0.430$, Chi-square test). Note that were only a few missing values, except for temperature, which was not measured for the Ngerenya-early sub-cohort.

3.1.2 The Assessment of the Infectious Reservoir of Malaria (AFIRM) Study

Table 3 presents the demographic characteristics for AFIRM. The median age of the study participants was 10 years (IQR 21 years). Though there were slightly fewer participants sampled in the dry season (96 participants) than the wet season (120 participants), there were no statistically significant differences in the numbers of participants in each of the age groups between the two seasons. When parasitaemia was detected using rapid diagnostic tests, there was higher parasite prevalence in the wet season (28.3% *vs* 12.5% in the wet and dry seasons, respectively, $p = 0.008$, Chi-square test). Conversely, when parasite detection was by microscopy, no significant differences in parasite prevalence were observed between the wet and dry season (16.7% *vs* 8.3% ($p = 0.108$, Chi-square test) for asexual parasites and 1% *vs* 3.3% ($p = 0.511$, Chi-square test) for gametocytes, respectively). Similarly, no differences were observed when detection was by molecular methods for all parasites (*18S* qPCR: 32.5% *vs* 41.7% ($p = 0.212$) and NASBA *18S*: 49.2% *vs* 50% ($p = 1$) in the wet and dry seasons respectively, Chi-square test) and gametocytes [NASBA *Pfs25:* 24% *vs* 25.8% in the wet and dry seasons respectively ($p = 0.874$, Chi-square test)]. There were no differences between the proportion of participants with the different sickle cell and α–thalassaemia genotypes between the wet and dry seasons.

TABLE 3 | Demographic characteristics of participants from the AFIRM cohort study.

	Season	
	Dry	**Wet**
Number of participants (N)	96	120
Sex: number of females (%)	61 (63.5)	61 (50.8)
Number per age group (%)		
0 - 5 years	27 (28.1)	45 (37.5)
6 - 15 years	33 (34.4)	39 (32.5)
>15 years	36 (37.5)	36 (30)
Temperature (°C), median (IQR)	36.6 (36.3 - 37.0)	36.6 (36.2 - 36.8)
Number RDT positive (%)	12 (12.5)	34 (28.3)
Parasite Prevalence		
qPCR (*18S*)	40 (41.7)	39 (32.5)
NASBA* (*18S*)	48 (50)	59 (49.2)
Asexual parasite prevalence - Microscopy (%)	8 (8.3)	20 (16.7)
Gametocyte prevalence (%)		
Microscopy	1 (1.0)	4 (3.3)
NASBA (*Pfs25*)	23 (24)	31 (25.8)
Sickle genotype (%)		
AA	77 (80.2)	102 (85)
AS	19 (19.8)	18 (15)
α - thalassaemia genotype (%)		
Normal	30 (31.3)	38 (31.7)
Heterozygous	51 (53.1)	56 (46.7)
Homozygous	15 (15.6)	26 (21.7)

**NASBA, nucleic acid sequence-based amplification.*

3.2 Relationship Between Parasite Density and the Covariates

Prior to evaluating the relationships between the magnitude of antibody responses to the candidate antigens and the covariates age, transmission intensity (sub-cohorts), season and sickle cell and α-thalassaemia genotypes, we first analyzed how these covariates relate to parasite densities. This was done to evaluate whether the influence of these covariates on parasite densities relates to their impact on the magnitude of antibody responses to the candidate antigens. For the KMLC, microscopically determined asexual parasite densities were higher in children aged between 0 – 5 years of age compared to those aged 6 – 5 years ($p = 0.019$, **Figure 1A).** In contrast, gametocyte densities were similar across age groups. There were no differences in both asexual and gametocyte densities among the different sickle (**Figure 1B**) and α-thalassaemia genotypes (**Figure 1C**) or across the subcohorts (**Figure 1D**). As few participants had patent gametocytaemia in the AFIRM cohort (**Table 3**), we compared sub-patent parasite densities by the covariates mentioned above. In this case, sub-patent gametocyte densities did not vary by age (**Figure 2A**) or by sickle trait (**Figure 2B**) and α-thalassaemia genotype (**Figure 2C**). However, parasite densities were higher in the wet season compared to the dry season, and this was statistically significant for both all parasites ($p = 0.015$) and gametocytes ($p = 0.013$, **Figure 2D**).

3.3 Seroprevalence to the Candidate Antigens

For seropositivity estimated from a population of malaria naïve individuals, there was a broad range in the seroprevalence estimates, ranging from 37.0% (95% CI: 30.6%, 43.9%) for PSOP1 to 77.8% (95% CI: 71.6%, 83.1%) for G377B 3D7 variant (**Table 4**). Seroprevalence to G377B PfKE04 was similarly high (70.4%, 95% CI: 63. 8%, 76.4%), and there was a significant association between participants who responded to G377B 3D7 and those who responded to G377B PfKE04 (χ^2 statistic = 110, p <0.0001; Chi-square test of independence with Yates' continuity correction, 2 x 2 tables provided in **Supplementary Table 2**). Seroprevalence to Pfs230-C was estimated as 64.2% (95% CI: 55.9%, 71.9%), with responses to both AMA1 and GE being the most prevalent at 87.9% (95% CI: 82.8%, 91.9%) and 88.3% (95% CI: 83.1%, 92.3%) respectively.

When seroprevalence was stratified by age, with age categorized as 0 – 5 years, 6 – 15 years and >15 years, there was a general trend towards increasing seroprevalence with age for all antigens being statistically significant for all except PF3D7_0208800 ($p = 0.064$, **Figure 3**). There was no evidence for a difference in seroprevalence between the dry and wet seasons

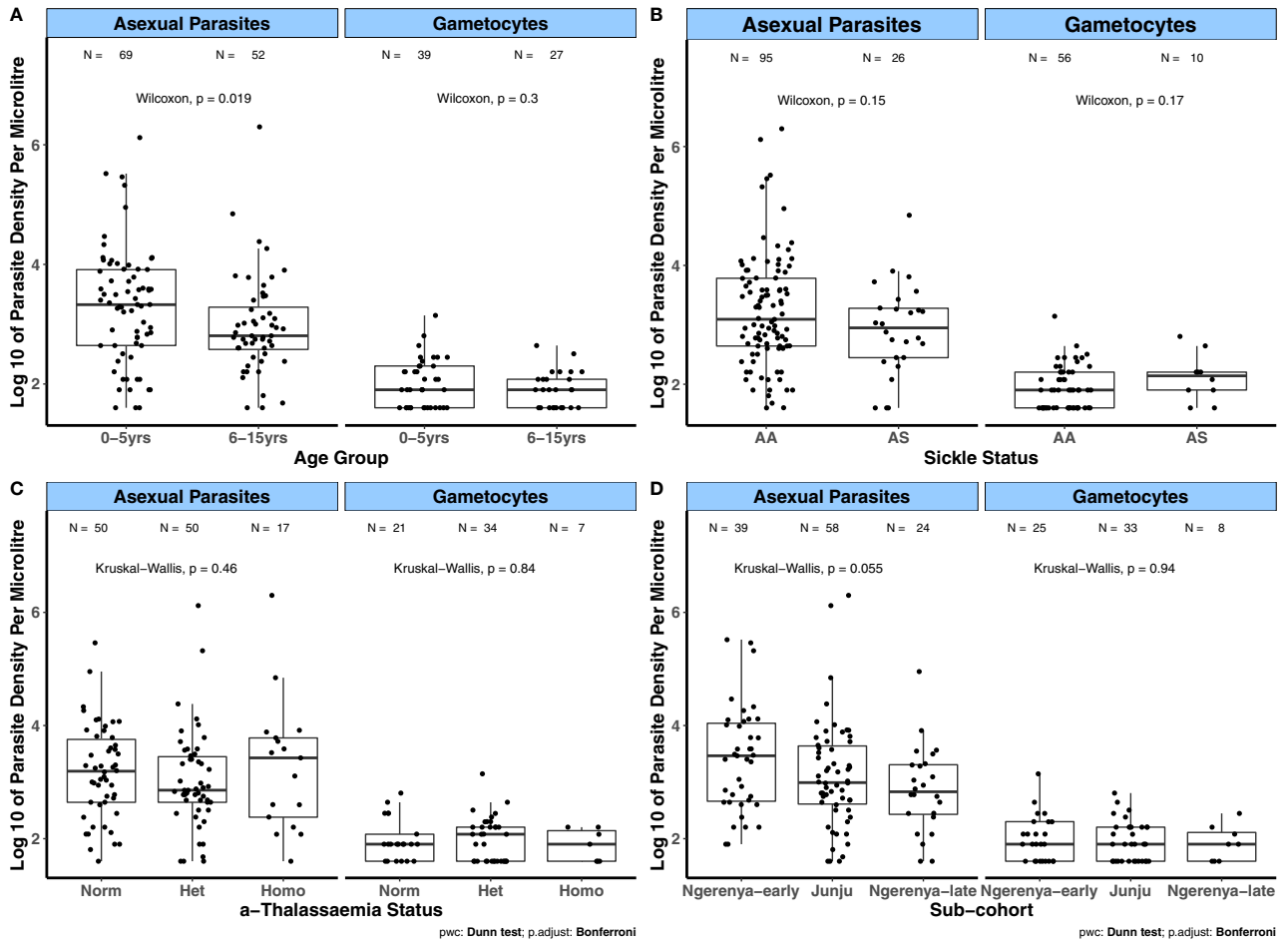

FIGURE 1 | Variation in patent parasite densities by factors associated with gametocyte carriage in the KMLC cohort. Asexual parasite and gametocyte densities by **(A)** Age group; **(B)** Sickle genotype; and **(C)** α-thalassaemia genotype. **(D)** Sub-cohort (moderate transmission (Ngerenya-early and Junju) and low transmission (Ngerenya-late)). Comparisons were carried out using the Wilcoxon test and Kruskal-Wallis test (post-hoc analysis after Kruskal-Wallis carried out using Dunn's test with Bonferroni correction). The number of parasite positive individuals (N) is indicated at the top of each graph. α-thalassaemia: Norm – normal, Het – heterozygous, and Homo – homozygous. The boxes of boxplots display the median bound by the first and third quartiles, with the whiskers depicting the lowest and highest values (excluding outliers). The dots indicate individual data points.

for any of the antigens (**Figure 4**). When seroprevalence was estimated using a subset of low responders as the seronegative population, the estimated seroprevalence was lower, particularly for the gametocyte antigens, likely reflecting the low ODs in the seropositive samples (**Supplementary Table 3**). Despite this, the trend towards increasing seroprevalence with age remained significant for all antigens, except for PEB-P (**Supplementary Figure 9**). Seroprevalence did not associate with season same as before (**Supplementary Figure 10**).

We also examined the dependency between antibody responses to the candidate gametocyte antigens, AMA1 and GE using a correlation matrix (**Supplementary Figure 11** and **Supplementary Table 4**). The strongest correlation was observed between seropositivity to the two G377B variants (Spearman's correlation ρ = 0.75, p <0.0001). Furthermore, there was a trend towards a stronger correlation between seropositivity to the different gametocyte antigens than between seropositivity to the gametocyte antigens and positivity to either AMA1 or GE. While seropositivity to AMA1 and GE did not strongly correlate with seropositivity to the gametocyte antigens, their strongest correlation was with each other (Spearman's correlation ρ = 0.51, p <0.0001).

3.4 Factors Associated With the Magnitude of Antibody Response: KMLC Cohort

Detailed results from the univariable and multivariable analysis of predictors of antibody levels are provided in **Supplementary Tables 5, 6**, with the results of the multivariable analyses discussed below.

Statistically significant associations between increased age and increased magnitude of antibody responses to AMA1, Pfs230-C, G377B 3D7 and G377B PfKE04 were observed. Furthermore, patent asexual parasitaemia was significantly associated with an increased

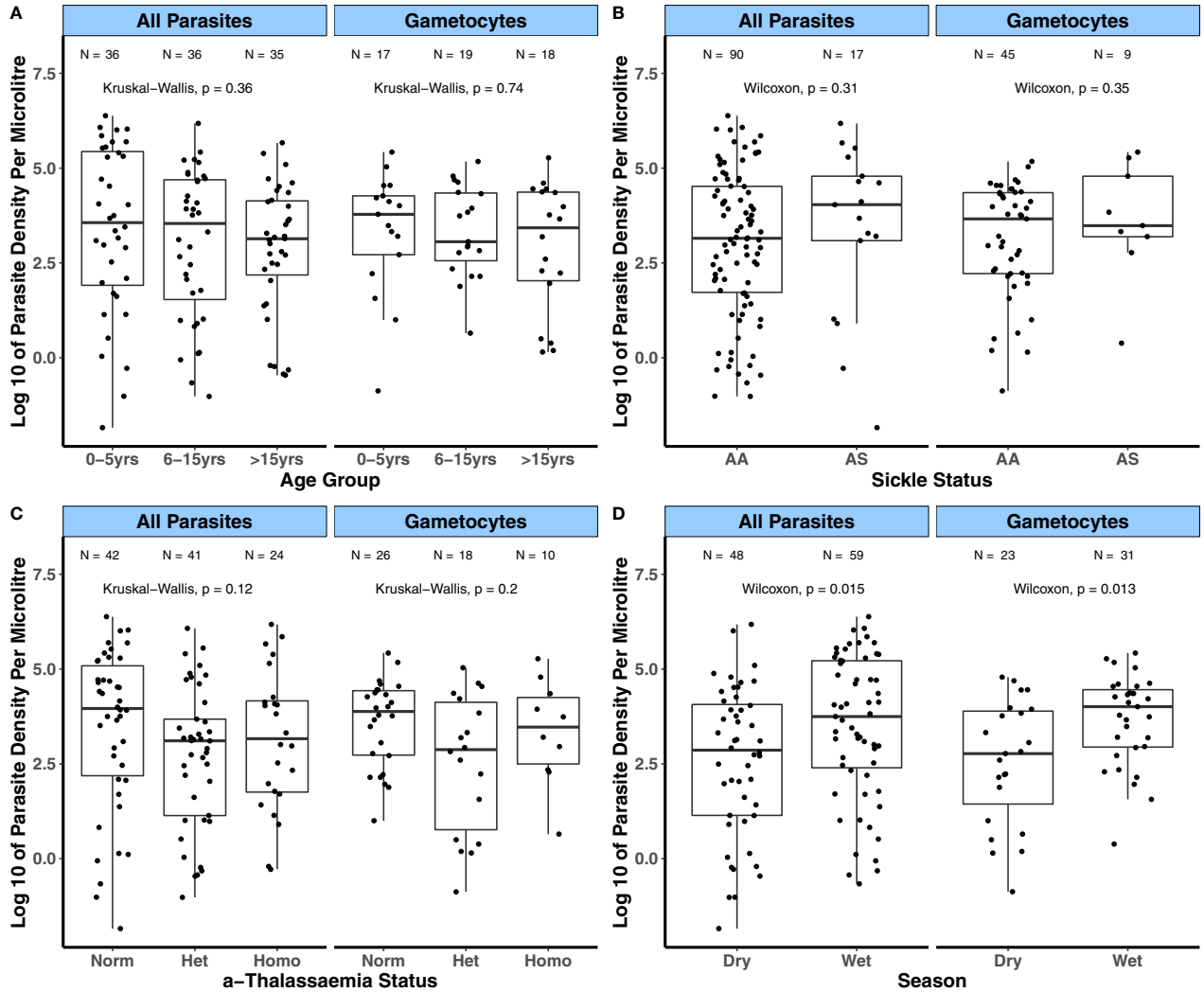

FIGURE 2 | Variation in sub-patent parasite densities by factors associated with gametocyte carriage in the AFIRM cohort. Asexual parasite and gametocyte densities by **(A)** Age group; **(B)** Sickle genotype; **(C)** α-thalassaemia genotype; and **(D)** Season with parasitaemia detected by *18S* NASBA (all parasites) and *Pfs25* NASBA (female gametocytes). Comparisons were carried out using the Wilcoxon test and Kruskal-Wallis test. The number of parasite positive individuals (N) is indicated at the top of each graph. α-thalassaemia: Norm – normal, Het – heterozygous, and Homo – homozygous. The boxes of boxplots display the median bound by the first and third quartiles, with the whiskers depicting the lowest and highest values (excluding outliers). The dots indicate individual data points.

magnitude of antibody responses to all antigens. Gametocytaemia, on the other hand, was only significantly associated with increased responses to AMA1, GE, PF3D7_1314500, PF3D7_0303900, and PF3D7_0208800 (p <0.05). No significant associations were observed between sickle cell trait and antibody levels for any of the antigens. Conversely, being heterozygous (estimate -0.24, 95% CI: -0.45, -0.03, p = 0.022) or homozygous (estimate -0.30, 95% CI: -0.57, -0.04, p = 0.025) for α-thalassaemia was associated with reduced responses to GE. Generally, transmission intensity did not appear to predict the magnitude of antibody response to the gametocyte antigens independent of parasite prevalence, except in the case of GE and G377B 3D7. Relative to the Junju sub-cohort (moderate transmission intensity), residing in the low transmission sub-cohort (Ngerenya-late) was associated with reduced immune responses to GE (estimate -0.30, 95% CI: -0.54, -0.06, p = 0.015) and G377B 3D7 (estimate -0.12, 95% CI: -0.23, -0.004, p = 0.043).

Both G377B 3D7 and G377B PfKE04 had similar associations between increasing age and increased magnitude of antibody responses, and increased antibody levels with concurrent parasitaemia. Additionally, R^2 values of the predictive models for both variants were similar (G377B 3D7 R^2 = 0.36, G377B PfKE04 R^2 = 0.36).

3.5 Factors Associated With the Magnitude of Antibody Response: AFIRM Cohort

Detailed results of the univariable and multivariable analyses are provided in **Supplementary Tables 7, 8**, with a summary of the results of the multivariable analyses provided below.

TABLE 4 | Seroprevalence of antibody responses to the gametocyte antigens, AMA1 and gametocyte extract.

Gene ID	Gene name[a]	N[b]	Median OD (Range)[C]	Seropositive	Prevalence (95% CI)
PF3D7_0209000	P230	148	0.22 (0.02, 2.50)	95	64.2 (55.9, 71.9)
PF3D7_1314500	–	216	0.35 (0.00, 3.34)	132	61.1 (54.3, 67.7)
PF3D7_0303900	–	216	0.22 (0.00, 2.81)	120	55.6 (48.7, 62.3)
PF3D7_0721700	PSOP1	216	0.24 (0.00, 2.53)	80	37.0 (30.6, 43.9)
PF3D7_0208800	–	216	0.37 (0.00, 2.49)	122	56.5 (49.6, 63.2)
PF3D7_1216500	MDV1	216	0.38 (0.00, 3.50)	126	58.3 (51.5, 65.0)
PF3D7_1250100	G377B 3D7	216	0.64 (0.00, 3.04)	168	77.8 (71.6, 83.1)
–	G377B PfKE04	216	0.59 (0.00, 2.94)	152	70.4 (63. 8, 76.4)
PF3D7_1133400	AMA1	215	1.00 (0.00, 3.68)	189	87.9 (82. 8, 91.9)
–	Gametocyte Extract	205	1.01 (0.00, 2.45)	181	88.3 (83.1, 92.4)

[a]*Where no parasite line is specified after the gene name, the gene sequence was based on the P. falciparum reference lab isolate 3D7. PfKE04 refers to a sequenced clinical isolate obtained from a clinical sample from the KMLC.*

[b]*Number of samples assayed for each antigen; Though the total AFIRM sample set consisted of 216 individuals, not all samples were tested for Pfs230-C or GE as antigen quantities were limited.*

[C]*OD, optical density; SD, standard deviation. The optical density is relative to the serum dilution used, with the gametocyte antigens tested at 1:200 (except for PEB-P and PSOP1 tested at 1:100), AMA1 tested at either 1:1000 or 1:2000 and GE 1:500.*

There was a statistically significant trend towards increasing magnitude of antibody responses with increasing age for Pfs230-C, AMA1 and GE, with only older age (>15 years of age) associated with an increased antibody response (p <0.05) to all other antigens except for PF3D7_0208800. Similar to the KMLC, parasitaemia was an important predictor of the magnitude of antibody response to the gametocyte antigens, with the exception of PSOP1. Both patent and sub-patent parasitaemia were associated with an increased magnitude of antibody responses to PF3D7_1314500, PF3D7_0303900, MDV1, G377B (both variants) and GE. To explore whether patent parasitaemia better predicted the magnitude of antibody responses to these antigens, a second

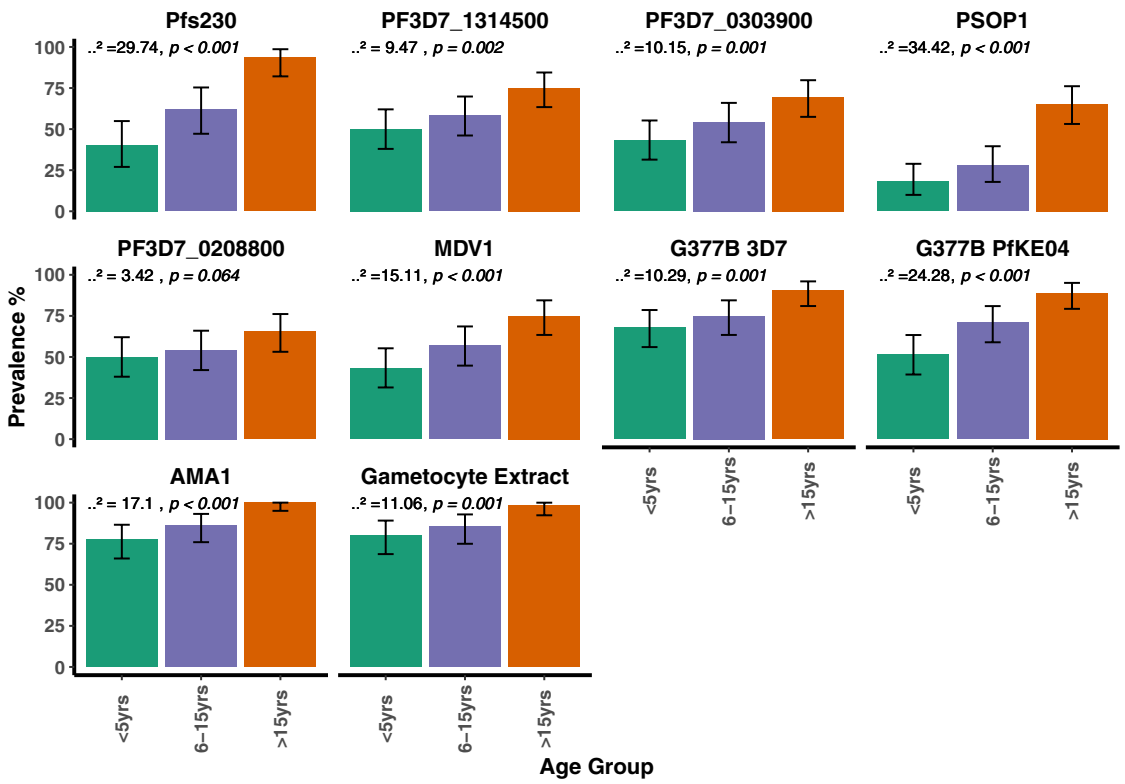

FIGURE 3 | Seroprevalence to the candidate antigens, AMA1 and gametocyte extract stratified by age group in the AFIRM Cohort. Bar plots showing the prevalence of antibodies to the candidate antigens, AMA1 and gametocyte extract within the different age categories. The Cochran-Armitage test for trend was used to analyze the relationship between seroprevalence and age; p values are presented at the top of each panel. Error bars show 95% binomial confidence intervals (Clopper–Pearson interval).

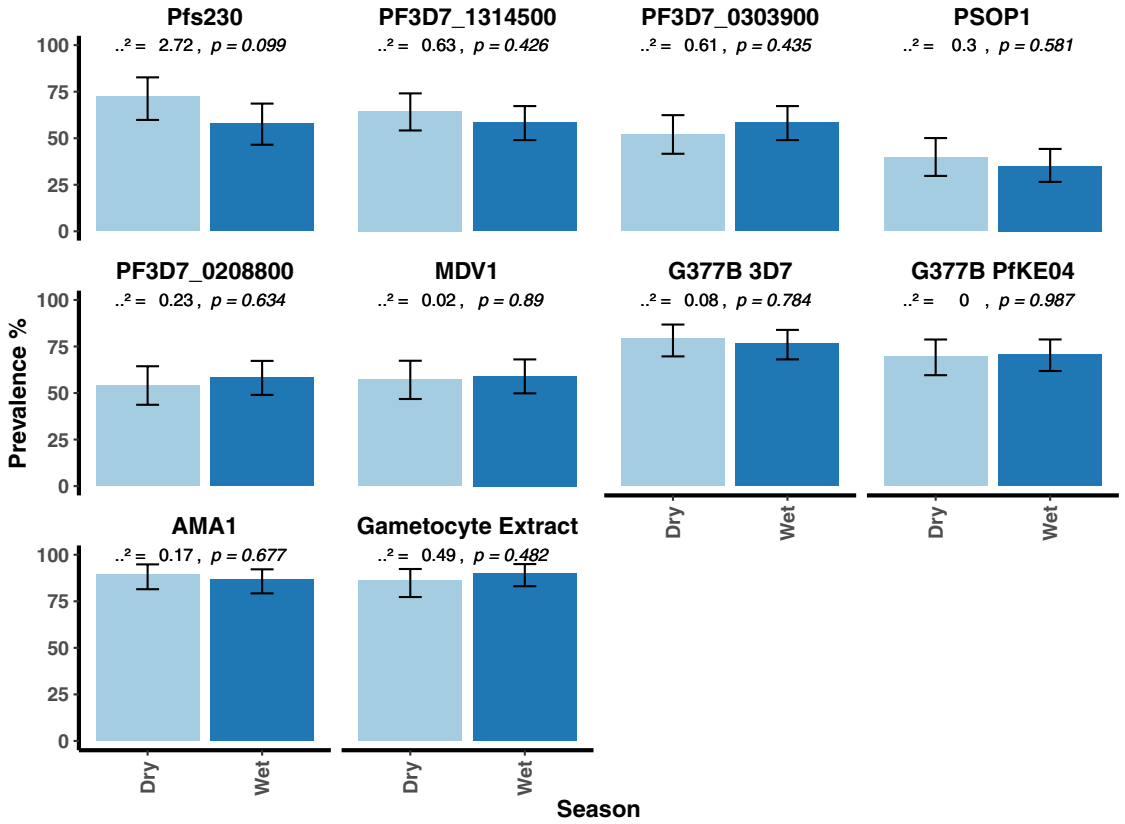

FIGURE 4 | Seroprevalence to the candidate antigens, AMA1 and gametocyte extract stratified by season in the AFIRM cohort. Bar plots showing the prevalence of antibodies to the candidate antigens, AMA1 and gametocyte extract in the dry and the wet seasons. A Chi-square-test was used to compare proportions between the dry and wet seasons. Respective *p* values are presented at the top of each panel. Error bars show 95% binomial confidence intervals (Clopper–Pearson interval).

analysis limited to positive parasite individuals was carried out (**Supplementary Tables 9, 10**). There was no indication that patent parasitaemia was a better predictor of the magnitude of antibody responses relative to sub-patent parasitaemia in this sub-analysis. Moreover, in the adjusted analysis, gametocytaemia did not independently predict the magnitude of antibody responses to the gametocyte antigens except for Pfs230-C (estimate 0.23, 95% CI: 0.02 – 0.44, $p = 0.030$).

Unlike in the KMLC, where no associations with sickle cell were seen, sickle cell trait was associated with a reduced magnitude of antibody response to PF3D7_0303900 in both the univariable and multivariable analysis (multivariable analysis: estimate -0.24, 95% CI: -0.42 – 0.05, $p = 0.013$). No associations among the α-thalassemia genotypes and antibody levels were seen. Similarly, no association was observed between sampling season and antibody levels for any of the antigens.

Similar to the KMLC, both variants of G377B had associations between increasing age and increased magnitude of antibody responses and between and concurrent parasitaemia and increased level of antibody responses. Additionally, the R^2 values of models predicting the magnitude of antibody responses to either antigen varied marginally (G377B 3D7 $R^2 = 0.34$, G377B PfKE04 $R^2 = 0.36$).

3.6 Factors Associated With the Breadth of Response

The mean (standard deviation) breadth of response to the eight gametocyte proteins was 4.5 (2.5) in the KMLC cohort and 5 (2.6) in the AFIRM cohort (**Figure 5**).

Adjusted linear regression analysis of factors associated with increased breadth indicated an association between increasing age and increased antibody breadth for both cohorts (**Tables 5** and **6**). Additionally, concurrent parasitaemia, whether asexual parasites or gametocytes, was also associated with increased breadth. No statistically significant associations with sickle and α-thalassemia genotypes, transmission intensity or season were observed.

4 DISCUSSION

We carried out seroepidemiological analyses on a set of relatively uncharacterized mature stage V gametocyte proteins, using sera from two cohorts of malaria-exposed individuals. The aim of this

work was to improve our understanding of naturally acquired immunity to sexual stage antigens that had previously been poorly characterized and hence identify new targets of naturally acquired immunity other than Pfs230-C and Pfs48/45. This is the first study of naturally acquired antibody responses in Kenya to use a panel of gametocyte antigens, as well as the first to describe patterns of seroprevalence to PF3D7_02080800.

Key determinants of malaria exposure as well as risk factors for gametocyte carriage were used to describe the dynamics of antibody responses to the candidate antigens. To place our results in the context of what is known, our list of candidates included the widely studied gametocyte/gamete antigen Pfs230-C (Bousema et al., 2007; Jones et al., 2015; Amoah et al., 2018; Oueadraogo et al., 2018; Stone et al., 2018) as well as two markers of parasite exposure AMA1 (Polley et al., 2004; Drakeley et al., 2005; Wong et al., 2014; Kangoye et al., 2016; Idris et al., 2017) and a crude extract prepared from a culture of mature gametocytes (GE) (Omondi et al., 2021). In this way, our study provides a more extensive profiling of the naturally acquired anti-gametocyte response than has been done in previous studies.

Antibodies to the panel of individual antigens tested were readily detected in the two cohorts analysed indicating that they are likely targets of naturally acquired immunity to gametocytes. Further investigations into their transmission-blocking activity are warranted, particularly for PF3D7_1314500, PF3D7_0303900 and PF3D7_0208800 that have not yet been tested as transmission-blocking vaccine (TBV) candidates, and for G377B that has similar patterns of association with Pfs230-C. Seroprevalence to the gametocyte antigens was quite varied. The highest seroprevalence was observed for G377B (both variants), with seroprevalence to Pfs230-C estimated as 64.2%. Lowest seroprevalence was seen for PSOP1. Though peak expression of PSOP1 may be observed at the ookinete stage, low-level expression may begin at the gametocyte stage as has been observed for its *P. berghei* homologue (Khan et al., 2005). Estimates of seroprevalence to Pfs230 from previous studies have varied widely, with variation attributable to differences in study design and immunoassay protocol such as 1) antigen coating concentration, 2) serum dilution, and 3) method used to determine the seropositivity cut-off (Muthui et al., 2019a). Therefore, seroprevalence estimates from different studies may not be directly comparable unless study designs and assay methodologies are similar, and this makes a case for standardized protocols for carrying out such studies. We found evidence for an age-dependent increase in seroprevalence to the candidate antigens. This may support the argument that malaria-exposed individuals develop some degree of long-lived antibody responses to sexual stage antigens with age (cumulative exposure). This may be important for natural boosting of TBV-induced antibody responses in the field, thereby making the TBV more efficacious.

Increased seroprevalence in the wet season has also been described (Ouédraogo et al., 2011; Skinner et al., 2015; Oueadraogo et al., 2018). However, it is important to note that these studies were done in areas with a pronounced difference in parasite prevalence between the dry and wet seasons, unlike the areas sampled in our study, and hence this may explain why we did not find evidence for an impact of season on seroprevalence.

In addition to seroprevalence, we also examined factors that may influence the magnitude of antibody responses to gametocyte antigens using data from two cohorts in separate analyses conducted using linear regression models. As with seroprevalence, there was evidence to suggest an influence of age on antibody levels, with the strongest evidence seen for Pfs230-C and G377B (both variants). In contrast, there was no evidence for an age-dependent increase in the magnitude of antibody responses to PF3D7_0208800. For PF3D7_0208800, concurrent parasitaemia was a better predictor of the magnitude of antibody responses rather than age. Concurrent parasitaemia was a strong predictor of the magnitude of antibody responses to all the candidate gametocyte antigens as well as to AMA1 and GE. Asexual parasitaemia is a crucial determinant of gametocyte carriage (Bousema et al., 2004; Ouédraogo et al., 2011; Koepfli et al., 2014) and hence can influence antibodies to gametocyte antigens. In addition to influencing the magnitude of antibody response, both age and concurrent parasitaemia were associated with an increased breadth of response to the gametocyte antigens. These findings are in line with what was reported by Osier et al. (2008) for a panel of asexual stage antigens.

Some studies have shown that haemoglobinopathies may increase gametocyte carriage (Trager and Gill, 1992; Lawaly et al., 2010). We hypothesized that this might correlate with an increase in the magnitude of antibody responses to the candidate antigens. However, we found that there was no clear evidence to suggest associations between either of the sickle or α-thalassaemia genotypes and the magnitude of antibody responses against the gametocyte antigens, as any trends observed were inconsistent across the two cohorts. A clearer understanding of the potential role of haemoglobinopathies in modulating responses to sexual stage antigens may be better discerned using longitudinal cohorts with frequent sampling over time.

For G377, variants based on 3D7 and a clinical isolate (PfKE04) were tested. Polymorphisms in malaria vaccine candidate antigens reduce their efficacy in the field (Genton et al., 2002; Laurens et al., 2013; Lyke, 2017; Draper et al., 2018); therefore, this is an essential factor to consider. Gametocyte antigens are thought to be relatively conserved (Doumbo et al., 2018). However, sequence variation does exist for some antigens. Acquah et al. (2017) investigated the impact of variation in the 6C region of Pfs48/45 (a single non-synonymous SNP present in 63.2% of the tested samples) and C0 region of Pfs230-C (nine base pair deletion present in 31% of the tested samples) on antibody responses. They found no impact of harbouring parasites with these variations on antibody responses to either antigen. In our study, seroprevalence did not differ between variants of G377B. Additionally, associations between either increasing age or concurrent parasitaemia and increased magnitude of antibody response were observed for both G377B variants. This could point to variant-transcendent responses to the G377B variants we evaluated; however, further experiments will be required to confirm this.

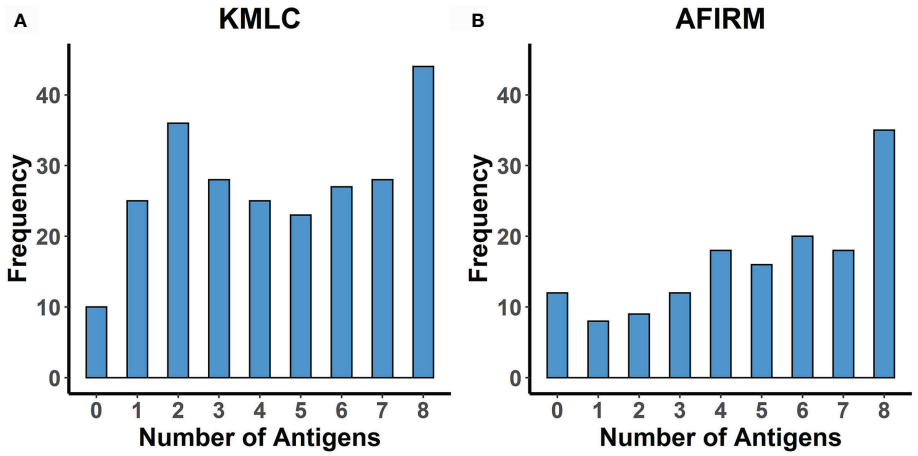

FIGURE 5 | | Breadth of response to the eight gametocyte proteins. Histograms showing the number of study participants seropositive to an increasing number of the proteins studied (limited to participants with responses measured to all eight proteins). **(A)** KMLC (N = 246) and **(B)** AFIRM cohort (N = 148).

While the two cohorts allowed independent evaluation of some of the factors relating to malaria exposure to predict responses to the gametocyte antigens, differences in study design and variables evaluated did not allow pooling of the studies; for instance, data on submicroscopic parasite carriage was not available in the KMLC cohort. Additionally, the cross-sectional nature of these cohorts did not allow discerning the relationship between recent versus prior parasite exposure and sexual stage antibody kinetics (Jones et al., 2015; Oueadraogo et al., 2018). Future longitudinal studies, including data on patent and sub-patent parasite densities (Ouédraogo et al., 2011) as well as infectivity at several time points (Stone et al., 2018), would be key to better defining both the kinetics of sexual stage immunity and its impact on infectivity. Moreover, such studies may need to

TABLE 5 | Linear regression analysis of the factors influencing the number of antigens recognized by the study participants – KMLC cohort.

Covariate	Univariable			Multivariable		
	Estimate	95% CI	*p* value	Estimate	95% CI	*p* value
Age Group						
0 - 5 years	Ref.	.	.	Ref.	.	.
6 - 10 years	0.11	0.03, 0.2	**0.0091**	0.11	0.03, 0.18	**0.0044**
11 - 15 years	0.1	-0.04, 0.25	0.1692	0.08	-0.06, 0.21	0.2528
Asexual parasite positive	0.29	0.22, 0.37	**<0.001**	0.23	0.14, 0.33	**<0.001**
Gametocyte positive	0.23	0.15, 0.3	**<0.001**	0.12	0.02, 0.22	**0.0217**
Sickle						
Normal	Ref.	.	.	Ref.	.	.
Heterozygous	0.04	-0.06, 0.15	0.4201	0	-0.09, 0.09	0.9724
α - thalassaemia						
Normal	Ref.	.	.	Ref.	.	.
Heterozygous	-0.04	-0.13, 0.05	0.3870	-0.01	-0.09, 0.07	0.8805
Homozygous	-0.03	-0.15, 0.09	0.6393	0.01	-0.1, 0.11	0.8794
Cohort						
Junju	Ref.	.	.	Ref.	.	.
Ngerenya-early	0.05	-0.11, 0.22	0.5226	-0.01	-0.13, 0.11	0.8459
Ngerenya-late	-0.17	-0.28, -0.05	**0.0048**	-0.06	-0.15, 0.03	0.2008

[a]*Parasitaemia as determined by microscopy.*

Ref., reference category. P values in bold are statistically significant (p<0.05).

TABLE 6 | Linear regression analysis of the factors influencing the number of antigens recognized by the study participants – AFIRM cohort.

Covariate	Univariable			Multivariable		
	Estimate	95% CI	*p* value	Estimate	95% CI	*p* value
Age Group						
0 - 5 years	Ref.	.	.	Ref.	.	.
6 - 10 years	0.12	0.02, 0.21	**0.0192**	0.11	0.02, 0.20	**0.0160**
11 - 15 years	0.31	0.21, 0.41	**<0.001**	0.31	0.22, 0.40	**<0.001**
Asexual parasite positive	0.23	0.14, 0.31	**<0.001**	0.18	0.09, 0.26	**<0.001**
Gametocyte positive	0.21	0.11, 0.31	**<0.001**	0.10	-0.002, 0.20	**0.0578**
Sickle						
Normal	Ref.	.	.	Ref.	.	.
Heterozygous	-0.06	-0.18, 0.05	0.2925	-0.06	-0.15, 0.04	0.2593
α - thalassaemia						
Normal	Ref.	.	.	Ref.	.	.
Heterozygous	-0.05	-0.14, 0.05	0.3634	0.004	-0.08, 0.09	0.9297
Homozygous	0.06	-0.06, 0.19	0.3303	0.08	-0.03, 0.19	0.1386
Season						
Dry	Ref.	.	.	Ref.	.	.
Wet	-0.01	-0.1, 0.07	0.7701	0.004	-0.07, 0.08	0.8964

[a]*Parasitaemia as determined by PCR (18S QT-NASBA for all parasites and Pfs25 mRNA QT-NASBA for female gametocytes).*
Ref., reference category. P values in bold are statistically significant (p<0.05).

incorporate a range of transmission intensities (from high to low transmission settings) to correctly define the impact of parasite exposure. Lastly, we analyzed a small panel of antigens which we endeavoured to produce as full-length ectodomains where possible. However, while we were able to identify previously defined patterns of association, a much larger panel of antigens may be required to wholly elucidate naturally acquired sexual stage immunity.

In summary, the key determinants of antibody responses to the gametocyte proteins explored in this analysis were concurrent parasitaemia and age. Notably, the strong association between age and antibody responses to Pfs230-C and G377B may serve as evidence for an age-dependent acquisition of antibody responses to some gametocyte antigens as seen with asexual stage antigens. However, whether this translates to functional immunological memory requires further investigation. Additionally, the ability of sub-patent parasitaemia to boost antibody levels highlights the importance of considering chronic infections when describing the dynamics of naturally acquired responses to gametocyte antigens. PF3D7_0303900, PF3D7_1314500, PF3D7_0208800 and GE appeared to have potential as serological markers of high-density gametocytaemia. This was especially true for PF3D7_0208800, where concurrent parasitaemia, rather than age, predicted the magnitude of response. The evidence presented here warrants further evaluation of their prognostic ability in longitudinal cohort studies as they may provide valuable tools for assessing the infectious reservoir. Furthermore, these antigens may have utility as indicators of populations where TBV implementation should be prioritised, or be used to monitor the success of TBV implementation.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository and accession number can be found here: Harvard Dataverse online repository through the link https://doi.org/10.7910/DVN/WL8TRW.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Kenya Medical Research Institute Ethics Review Committee (reference numbers KEMRI/SERU/SSC2574 - AFIRM cohort, and KEMRI/SERU/CGMRC//3149 and KEMRI/SERU/SSC1131 - KMLC cohort). Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

MM and MK conceived and designed the study. MM, ET, BRO, CK, WM, and HN performed the experiments. MM, KM, and BO performed the statistical analysis. MM wrote the paper. JW, TB, CD, AB, KM, MK, and PB set up the cohorts of participants and participated in the drafting of the manuscript. All authors contributed to the preparation of the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2021.774537/full#supplementary-material

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Profiling the Quality and Quantity of Naturally Induced Antibody Responses Against Pfs230 and Pfs48/45 Among Non-Febrile Children Living in Southern Ghana: A Longitudinal Study

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A clear understanding of the properties of naturally induced antibody responses against transmission-blocking vaccine candidates can accelerate the understanding of the development of transmission-blocking immunity. This study characterized the naturally induced IgG responses against two leading transmission-blocking vaccine antigens, Pfs230 and Pfs48/45, in non-febrile children living in Simiw, Ghana. Consecutive sampling was used to recruit 84 non-febrile children aged from 6 to 12 years old into the 6-month (November 2017 until May 2018) longitudinal study. Venous blood (1 ml) was collected once every 2 months and used to determine hemoglobin levels, *P. falciparum* prevalence using microscopy and polymerase chain reaction, and the levels and relative avidity of IgG responses against Pfs230 and Pfs48/45 using indirect ELISA. IgG levels against Pfs230 and Pfs48/45 decreased from the start (November) to the middle (January) and end (March) of the dry season respectively, then they began to increase. Participants, especially older children (10–12 years old) with active infections generally had lower antibody levels against both antigens. The relative avidities of IgG against both antigens followed the trend of IgG levels until the middle of the dry season, after which the relative avidities of both antigens correlated inversely with the antibody levels. In conclusion, although IgG antibody levels against both Pfs48/45 and Pfs230 began to increase by the early rainy season, they were inversely correlated to their respective relative avidities.

Keywords: antibody levels, relative avidity, gametocyte, *P. falciparum*, polymerase chain reaction (PCR)

Abbreviations: IgG, immunoglobulin G; PCR, polymerase chain reaction; HB, hemoglobin; aa, Amino Acid; DNA, Deoxyribonucleic acid; wConc, weighted concentration; PD, parasite density; RAI, relative avidity index; yrs, years.

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INTRODUCTION

The cycle of malaria transmission can be broken by the development of an immune response that arrests the progress of the infectious stages of the parasite, including gametocytes. *Plasmodium falciparum* antigens Pfs230 and Pfs48/45 are among the most widely characterized gametocyte surface proteins (Arredondo et al., 2012; Jones et al., 2015; MacDonald et al., 2016; Acquah et al., 2017). The Pfs230 is a 3,135 amino acid (aa) protein containing 14 s48/45 6-Cys domains (Williamson & Kaslow, 1993) and Pfs48/45 is a 448 aa protein containing 3 s48/45 6-Cys domain (Outchkourov et al., 2008). Antibodies against both Pfs230 and Pfs48/45 antigens possess transmission-reducing activities and are thus able to prevent the completion of the sporogonic life cycle of the malaria parasite within the mosquito vector (Kumar et al., 1995; Bousema et al., 2006). Enhanced knowledge of the properties of naturally induced antibody responses against these antigens can help accelerate the understanding of malaria transmission-blocking vaccine (MTBV) candidate development. Children have been identified to efficiently harbor gametocytes (Lamptey et al., 2018) and make them a relevant group to study the quality (avidity) and quantity (level) of naturally induced IgG responses against gametocyte antigens.

Although there are a number of different antibody types, IgG and IgM occurred to be the most commonly examined types (Leoratti et al., 2008; Zakeri et al., 2011; Mayor et al., 2018) which have also been found to keep a *P. falciparum* infection under control (Dodoo et al., 2008). Usually, an encounter between a B cell and parasite antigen activates the B cell with the help of T cell, to proliferate and differentiate into plasma cells. Plasma cells then release IgM primarily and IgG secondarily into circulation (Alam et al., 2013), binding to the target antigen. In many instances, repeated exposure of a B cell to the same antigen results in an increase in the total bond strength of the antibody-antigen complex (avidity) (Howitt et al., 2009; Murugan et al., 2018).

Protection against *P. falciparum* parasites is known to be associated with high antibody avidity rather than just high antibody levels (Bachmann et al., 1997; Ross et al., 2001). However, in some instances, high antibody levels have been found to compensate for low antibody avidity and maintain an individual's acquired immunity (Ssewanyana et al., 2017). The immunity that results from repeated exposure to the sexual stages of the malaria parasite (malaria transmission-blocking immunity) can prevent the mosquito from becoming infectious and thus reduce malaria transmission rather than protect the individual directly (Healer et al., 1999; White et al., 2018).

This study sought to characterize the properties (levels and relative avidities) of naturally-induced antibody responses against two transmission-blocking vaccine candidates, Pfs230 and Pfs48/45 among non-febrile children in southern Ghana.

METHODS

Ethics, Study Site, Population, and Sampling

Ethical clearance for this study (NMIR-IRB CPN 024/14-15) was obtained from the Institutional Review Board (IRB) of Noguchi Memorial Institute for Medical Research (NMIMR). Written informed consent was obtained before enrolling participants for this study.

The study was conducted in Simiw (**Figure 1**); a peri-urban community in the Komenda–Edina–Eguafo–Abrem (KEEA) Municipal Assembly of the Central Region of Ghana. Malaria transmission in Simiw is usually perennial and overlaps with the rainy season peaking in-between May and July. The dry season

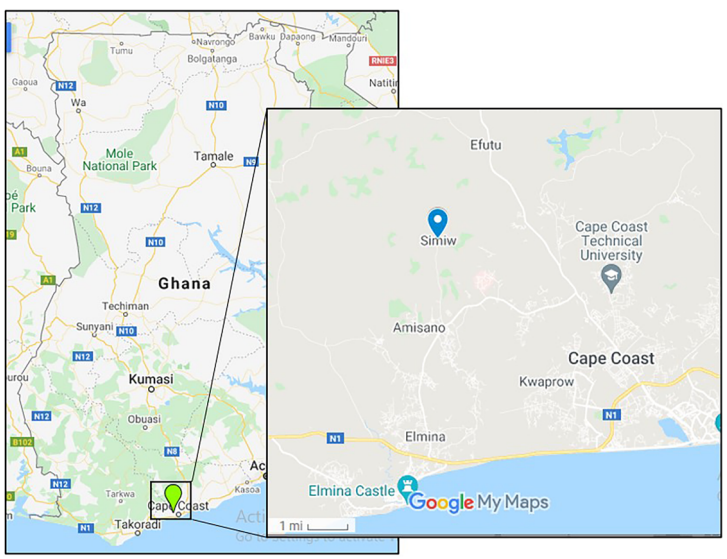

FIGURE 1 | A map of Ghana showing the study site (Simiw) in the enlarged area. The map was generated using Google maps.

however begins in November and ends in April (Acquah et al., 2020). Study participants were pupils of the Simiw M/A Basic School-aged from 6 to 12 years.

A total volume of 1 ml of the venous blood sample was collected from each of the 84 study participants once every 2 months beginning November 2017 until May 2018. Prior to sample collection, the axillary temperature was measured using a digital thermometer. Thick and thin smears were prepared according to WHO guidelines (Organization, 2016). Hemoglobin levels were determined using a drop of whole blood (15 µl) and the Urit 12 HB meter (URIT Medical Electronic Co. Ltd., China). Three 50 µl drops of whole blood were also spotted onto Whatmann #3 filter paper (GE Healthcare, USA). The remaining blood was separated into plasma and packed cells and each stored at −20°C.

Estimation of Parasite Density

The thick and thin blood smears were processed according to WHO guidelines (Organization, 2016) and as previously described (Amoah et al., 2019b). The smears were observed under 100× oil immersion objectives by two independent trained microscopists and any inconsistency in deciding a positive or negative slide was settled by the reading of a third microscopist. Parasite density was estimated by multiplying the number of parasites counted per 200 white blood cells (WBCs) by 40 based on the assumption that 1 µl of blood contains 8,000 WBCs.

DNA Extraction

The Saponin/Chelex DNA extraction method was used as previously described (Amoah et al., 2018). Briefly, two 3 mm discs were punched out of the dried filter paper blood spot into a 1.5 ml Eppendorf tube. A measure of 1 ml solution of 1× phosphate-buffered saline and 50 µl of 10% saponin was added to each tube, vortexed, and kept overnight at 4°C. The discs were washed twice with 1 ml phosphate-buffered saline and kept in 30 µl of 20% chelex and 70 µl of Dnase/Rnase water was incubated at 95°C for 10 min with intermittent vortexing. Finally, the tubes were spun for 6 min at 13,000 rpm and the supernatant containing the extracted gDNA was transferred into a sterile 0.5 ml microfuge tube and preserved at −20°C.

Determination of *P. falciparum* by Polymerase Chain Reaction

Nested polymerase chain reaction, was used to amplify the 18S *rRNA* gene of *P. falciparum* as previously described (Amoah et al., 2019b). The Nest 1 reaction mixture contained 80 nM of primers (rPLU5/rPLU6), 5 µl DNA template, 1× polymerase chain reaction buffer, 167 nM dNTPs, 2.5 mM $MgCl_2$, and 1 U of OneTaq DNA polymerase, which totaled up to 15 µl mixture. The nest 2 reaction mixture contained 133.33 nM of primers (rFAL1/rFAL2) and 0.5 µl of the nest 1 product. The cycling conditions were set as follows; initial denaturation (95°C for 5 min), then 35 cycles of denaturation at 94°C for 30 s, annealing at 55 and 58°C (for nest 1 and nest 2 respectively) for 1 min and an extension at 68°C for 1 min with a final extension at 68°C for 5 min. The nest 2 polymerase chain reaction products were gel electrophoresed and visualized using UV illumination.

Determination of IgG Levels by Indirect Enzyme-Linked Immuno-Sorbent Assay (ELISA)

The IgG levels were determined according to previously described protocols (Amoah et al., 2019a). The Pfs230 antigen used in this study was the prodomain, amino acid (aa) residues 443 to 590 while the Pfs48/45 antigen comprised of the C-terminal 6C region (aa 291–428); both of which were expressed in *Lactococcus lactis* (Acquah et al., 2017). The negative control samples comprised of pooled plasma from malaria naïve individuals. The plasma used for plotting the standard curve and the positive control sample comprised of pooled plasma from individuals who were previously identified as containing high levels of IgG against these two antigens (Arredondo et al., 2012; Jones et al., 2015; MacDonald et al., 2016; Acquah et al., 2017). The Maxisorp plates (Nunc, Thermo Fisher) were coated with 100 µl of antigen (Pfs48/45 or Pfs230) diluted to 1 µg/ml in carbonated buffer (pH of 9.2) and the plasma samples were diluted 1:200 in 1% skimmed milk in 1× PBS containing 0.5% Tween 20. The rest of the procedure was followed through to the reading of the plates using the ELx808 plate reader (BioTek) set at 450 nm as previously described (Amoah et al., 2019a).

Determination of Relative Avidity by ELISA

The relative avidity of IgG in each sample was determined as previously described (Amoah et al., 2019a). A procedure similar to the ELISA described above was used with an extra step incorporated after the plasma incubation step. Briefly, each plasma sample was added to four wells of the plate, after the set incubation time of 1 h, a 100 µl solution of 2.4 M sodium thiocyanate was added to two of the four wells for 15 min. After this incubation step, the wells of the plates were washed and the procedure followed through with the secondary IgG incubation step to the stop reaction exactly as with the indirect ELISA described above.

Data Analysis

The OD values obtained from the plate reader were converted into weighted concentrations (wConcs) using ADAMSEL (Ed Remarque, BPRC). Graphs were plotted using GraphPad Prism version 6 and Microsoft Excel. The Kruskal–Wallis test and Dunn's multiple comparison *post-hoc* tests were used to assess statistical significance between median values of quantitative data between age groups (overall, ≥10 yrs and <10 yrs) and time points (November 2017, January 2018, March 2018 and May 2018). Within-individual variation of the antibody and avidity levels across the study period was assessed with the Friedman test. Association between microscopic and submicroscopic *P. falciparum* prevalence was determined by chi-square test and correlation between IgG responses against Pfs230 and Pfs48/45 were determined using Spearman's rank correlation (using GraphPad version 6). Linear regression models were fitted for the avidity of antibody levels in the final visit (May 2018) using the number of infections by microscopy, PCR, or both as the predictor variables. The association of *P. falciparum* carriage in previous months with that of May and their corresponding odds ratio was also determined using Graphpad prism. Statistical significance was determined at

P <0.05. The relative avidity index (RAI) for Pfs230 IgG, Pfs48/45 IgG was calculated as the ratio of the IgG concentration of the sodium thiocyanate-treated sample to the IgG concentration of the corresponding untreated sample multiplied by 100.

Plasma samples were assigned seropositive for antibodies against an antigen if the concentration of the antibodies in the sample was greater than the average plus two times the standard deviation of that for the negative control sample (pooled malaria-naïve plasma).

RESULTS

Clinical Characteristics of the Study Participants

The study recruited non-febrile school children aged from 6 to 12 years residing in the Simiw community in the Central Region of Ghana. A total of 84 children with a median (IQR) age of 9 (8–11) years were recruited in November 2017 and followed until May 2018. A total of 43 of the children were below 10 years old. No significant differences were observed in the median haemoglobin levels (Kruskal–Wallis statistic = 5.250, *P* = 0.1544) and temperature (Kruskal–Wallis statistic = 5.966, test *P* = 0.1133) of the children at all the four-time points (**Table 1**).

Asexual parasite prevalence estimated by microscopy was highest in March 2018 (36.9%) and lowest (7.14%) in May 2018, with the highest and lowest median parasite densities detected in May 2018 and January 2018 respectively (**Table 2**). Parasite prevalence estimated by polymerase chain reaction was highest (74.4%) in May and lowest (27.3%) in January. Variation in asexual parasite density was not statistically significant over the four time points (*P* = 0.2303, Kruskal–Wallis statistic = 4.305). None of the samples used in this study tested positive for gametocytes by microscopy (**Table 2**) at any time point. Parasite carriage in May was significantly associated with that of March (Fisher's exact text, p = 0.0118) with participants who carried parasites in March having odds of 5.3 of being infected in May (**Additional File 1**).

Dynamics of Antibody Responses Against Pfs48/45

The overall median IgG level against Pfs48/45 did not show statistically significant variation from November 2017 to March 2018. In May, however, overall anti-Pfs48/45 IgG levels increased significantly compared to all preceding months of the dry season assessed (p <0.0001). The median Pfs48/45 IgG levels in both the young children (<10 yrs) and older children (≥10 yrs) did not vary significantly from November 2017 to March 2018 except the period from January to March, where IgG levels of the young children decreased significantly (P = 0.867). In May, however, median anti-Pfs48/45 antibody levels increased significantly relative to March levels for both young children (P<0.001) and older children (P<0.01) (**Figure 2A**). Between the two age groups, older children had higher antibody levels than younger children in only March 2018, the second half of the dry season, but statistically the same for the other months assessed.

TABLE 1 | Characteristic of the study participants.

	November 2017	January 2018	March 2018	May 2018	*P*-value
Number of children	84	84	84	84	
Children <10 yrs	43	43	43	43	
Children ≥10 yrs	41	41	41	41	
Hb (g/dl)					
Median	11.35	11.50	11.60	11.60	0.1544
IQR	10.30–12.20	10.70–12.20	10.70–12.30	10.80–12.50	
Temp (°C)					
Median	36.70	36.40	36.40	36.40	0.1133
IQR	36.13–36.9	36.3–36.8	36.10–36.7	36.0–36.70	

Hb, Hemoglobin, Temp, Temperature; IQR, Interquartile range; yrs, years.

TABLE 2 | P. *falciparum* parasite density and prevalence by microscopy and polymerase chain reaction.

	November 2017	January 2018	March 2018	May 2018	*P*-value
Microscopy					
Median PD/µl (IQR)	1040.0 (300.0–4680)	280.0 (160.0–360.0)	880.0 (400.0–2440)	1920.0 (80.0–4330)	0.2303
Asexual parasite prevalence (% (n/N))	26.2 (22/84)	8.3 (7/84)	36.9 (31/84)	7.14 (6/84)	
Gametocyte prevalence (% (n/N))	0 (0/84)	0 (0/84)	0 (0/84)	0 (0/84)	
Polymerase chain reaction					
Total parasite prevalence (% (n/N))	27.3 (23/84)	46.4 (39/84)	42.9 (36/84)	74.4 (60/84)	

PD, Parasite density (reported per µl blood); IQR, Interquartile range; n, number of samples confirmed positive for P. falciparum by polymerase chain reaction; N, total number of samples tested for polymerase chain reaction.

The median relative avidities of anti-Pfs48/45 IgG in all the study participants (overall and age stratified) did not vary statistically from November to January. In March 2018 however, median relative avidity increased significantly for all children (P <0.001). Transitioning from March to May produced a significant decrease in the overall median relative avidities (P = 0.0292) although it was statistically unchanged for both the young children (P = 0.106) and older children (P = 0.103) (**Figure 2B**) sub-groups. Anti-Pfs4845 antibody levels but not avidity correlated positively (r = 0.282, P = 0.0095) with age in only March, the end of the dry season (**Additional File 1**). Within individuals, both the level and relative avidity of anti-Pfs48/45 IgG varied significantly between the various time points (P <0.0001 for both) (**Additional File 1**)

Dynamics of Antibody Responses Against Pfs230

The median levels of anti-Pfs230 antibodies of the overall participants significantly increased in March (P = 0.001) and in May (P = 0.0125). In younger children, median IgG levels against Pfs230 increased significantly in only May (P = 0.014). In older children, however, median anti-Pfs230 antibody levels had a statistically significant increase in March (P = 0.002) and remained the same in May (P = 0.250). Median antibody levels were higher for young children than older children in November 2017, the beginning of the first half of the dry season (P = 0.036), while the reverse occurred in March 2018 (P = 0.0007), close to the end of the second half of the dry season (**Figure 3A**). There were no statistically significant differences in the median relative avidities for the participants as a whole across the four time points (P = 0.0673). The relative avidity of anti-Pfs230 IgG in older children (>10 yrs) did not show any statistically significant variations (P = 0.325) over the duration of the study (**Figure 3B**). In younger children (<10 yrs) however, the median relative avidities of anti-Pfs230 IgG dropped significantly in May (P <0.001), to values lower than recorded in the older children (**Figure 3B**). Similar to Pfs48/45, anti-Pfs230 antibody levels correlated positively with age (r = 0.358, P = 0.008) but the relative avidity did not. In May however, both the levels and relative avidities of IgG against Pfs230 were positively correlated (r = 0.225, P = 0.0397 and r = 0.236, P = 0.0305, respectively) with age. The anti-Pfs230 antibody levels and relative avidities also varied significantly within individual participants (P = 0.0049 and P = 0.026, respectively) (**Additional File 1**)

Seroprevalence of Antibodies Against Pfs230 and Pfs48/45

Percentage seroprevalence of IgG antibodies against both Pfs48/5 and Pfs230 of all the participants had a similar trend, decreasing from the beginning of the dry season (November 2018) to the middle of the dry season (January) after which it increased again by the end of the dry season (March) and the

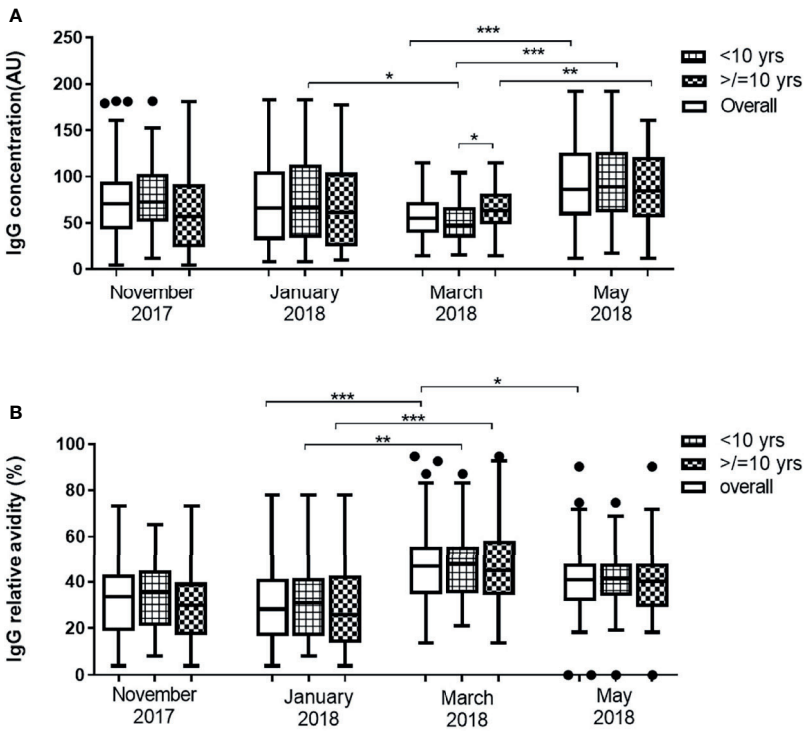

FIGURE 2 | Changes in Pfs48/45 IgG level **(A)** and avidity **(B)** in 43 children <10 years and 41 children ≥10 years over the dry season spanning November 2017 to May 2018. Yrs, years. The extent of statistically significant difference in measurements indicated by *, ** and *** represent P <0.05, P <0.01, and P <0.001 respectively.

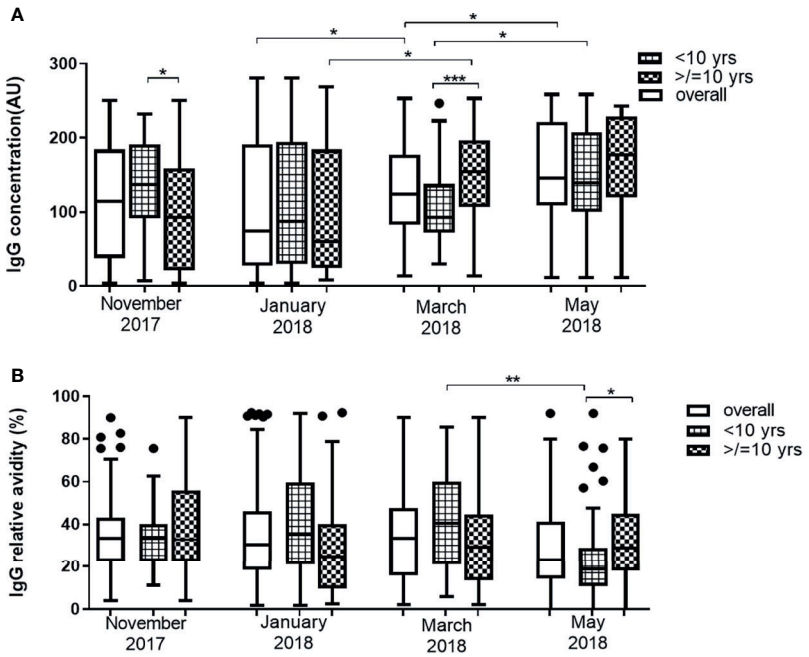

FIGURE 3 | Changes in Pfs230 IgG level **(A)** and avidity **(B)** in 43 children <10 years and 41 children ≥10 years over the dry season spanning November 2017 to May 2018. The extent of statistically significant difference in measurements indicated by *, ** and *** represent P <0.05, P <0.01, and P <0.001 respectively.

early rainy season (May) (**Figure 4**). The observed trend of overall seroprevalence for both antigens was similar to that of median parasite densities of participants. The percentage seroprevalence of both the younger children and older children categories followed the same trend as the overall participants. In November and January, younger children had a higher seroprevalence of IgG against both antigens than older children. In March and May, however, older children had a higher seroprevalence of antibodies against Pfs230 than the younger children. For anti-Pfs48/45 however, older children had a higher Percentage seroprevalence than younger children in only March (**Figure 4**).

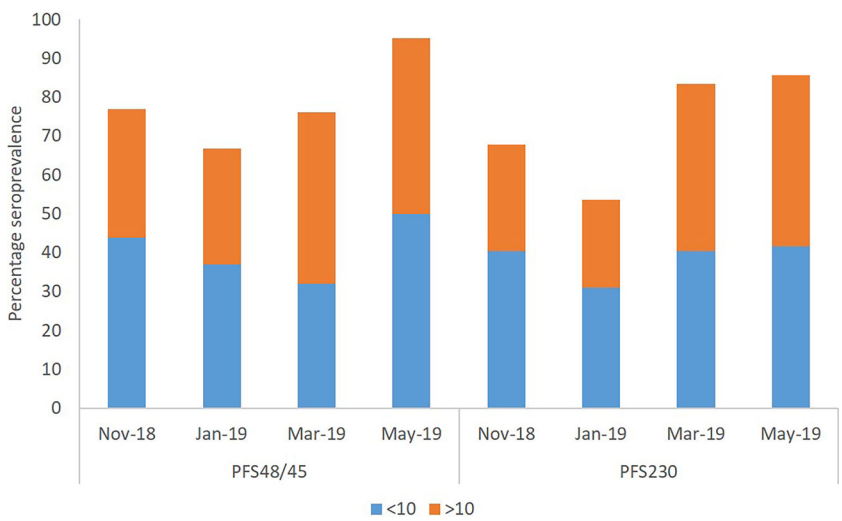

FIGURE 4 | Seroprevalence of IgG antibodies against Pfs230 and Pfs48/45. Immune responses of participants were considered seropositive when their antibody concentrations were higher than that of the average plus twice the standard deviation of the negative control sample's concentration.

Dynamics of Antibody Responses in the Presence or Absence of Active *P. falciparum* Infection

In the overall dataset, anti-Pfs48/45 IgG levels were higher in children without active infections in November (P = 0.027) and January (p = 0.019). In March however, the IgG levels in children without active infections dropped to similar levels as children with active infections, whose IgG levels remained unchanged from November to March. In May, the early parts of the rainy season anti-Pfs48/45 IgG in both children with and without active infections rose significantly relative to March levels (P <0.001 for both months) (**Figure 5A**).

Children with and without active infections had similar relative anti-Pfs48/45 IgG avidity at all assessed time points. The relative avidities were unchanged between November and January but increased significantly in March (P <0.001) and remained at the same level in May (**Figure 5B**) for both children with and without active infection.

Each of the two age groups (children <10 and ≥10 yrs) were sub-grouped into those with active infections (parasites detected by polymerase chain reaction, PCR+) and those without active infections (no parasites detected by polymerase chain reaction, PCR−) and their anti-Pfs48/45 and anti-Pfs230 IgG antibody levels and avidities compared. It was observed that the antibody responses (both IgG levels and avidities) in the two groups of children (with and without active infection) were similar throughout the study period except in January when the Pfs48/45 IgG levels of young children without active infections was higher than their counterparts with active infections ($P = 0.0112$) (**Figure 5A**). In May, when younger children without an active infection had higher relative avidity (P = 0.019) than older children with active infection (**Figure 5B**).

Overall, children with active infections had similar anti-Pfs230 IgG levels as those without active infections at all-time points. Anti-Pfs230 IgG levels however increased in children without active infection from January, mid dry season, to March which is towards the end of the dry season. Children with active infections in May had higher anti-Pfs230 IgG levels than in March (P = 0.0041). Children with and without active infections had similar median relative avidities at all-time points in all children as a whole or in the two age categories (**Figure 6B**). In March, older children (≥10 yrs) without active infections had

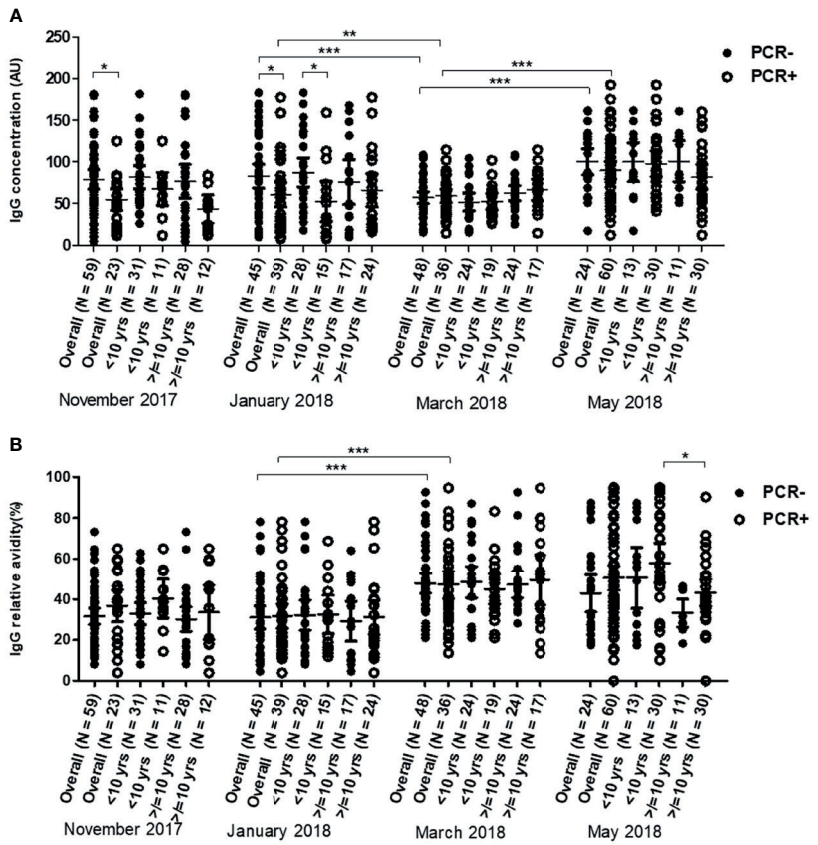

FIGURE 5 | Variations in of anti-Pfs48/45 IgG levels **(A)** and Relative avidities **(B)** in young children (<10 years) and older children (≥10 years) with (positive polymerase chain reaction test result) and without (negative-polymerase chain reaction test result) active *P. falciparum* infections over the dry season from November 2017 to May 2018. The number above each of the aligned dot plots represents the number of individuals in the group. The extent of statistically significant difference in measurements indicated by *, ** and *** represent P <0.05, P <0.01, and P <0.001 respectively.

significantly higher anti-Pfs230 IgG levels than their age mates with active infections (P = 0.040) and young children (<10 yrs) with (P = 0.0003)) or without active infections (P = 0.00135) (**Figure 6A**).

In May, the median relative avidity of Pfs230 IgG in older children (≥10 yrs) without active infections was significantly higher (P = 0.0263) than in younger children <10 yrs with active infections (**Figure 6B**). Older children with active infection had significantly lower relative avidity of anti-Pfs230 IgG compared to their age mates without active infections (P = 0.0378) or young children with active infections (P = 0.0315) in January. In May, however, the median relative avidity of older children with active infections rose to that of older children without active infection whose median relative avidity was significantly higher than young children without active infection (**Figure 6B**). The individual antibody levels and relative avidities used in this section can be found in **Additional File 1**. Regressional analyses showed that the number of times an individual tested positive for parasites by PCR or microscopy were not significant predictors of the antibody levels and avidities against the two antigens. Consequently, there was no statistical difference between the number of times a person had been infected during our visits and the anti-Pfs230 parasite and anti-Pfs48/45 antibody levels and avidity (**Additional Files 1 and 2**).

Association Between IgG Responses Against Pfs48/45 and Pfs230

In November, no correlation was identified between the measured IgG levels and avidity against Pfs48/45. Moderate and positive correlations were identified in January which changed to a weak inverse correlation in both March and May. All the correlations identified other than in November were significant.

Significant negative correlations existed between IgG levels and avidity against Pfs230 throughout the study period. The correlation was strong in the first half of the study period but weak towards the peak season (in the second half of the study period) (**Table 3**).

DISCUSSION

Although there are numerous reports on the development of immunity against the disease-causing asexual *P. falciparum*

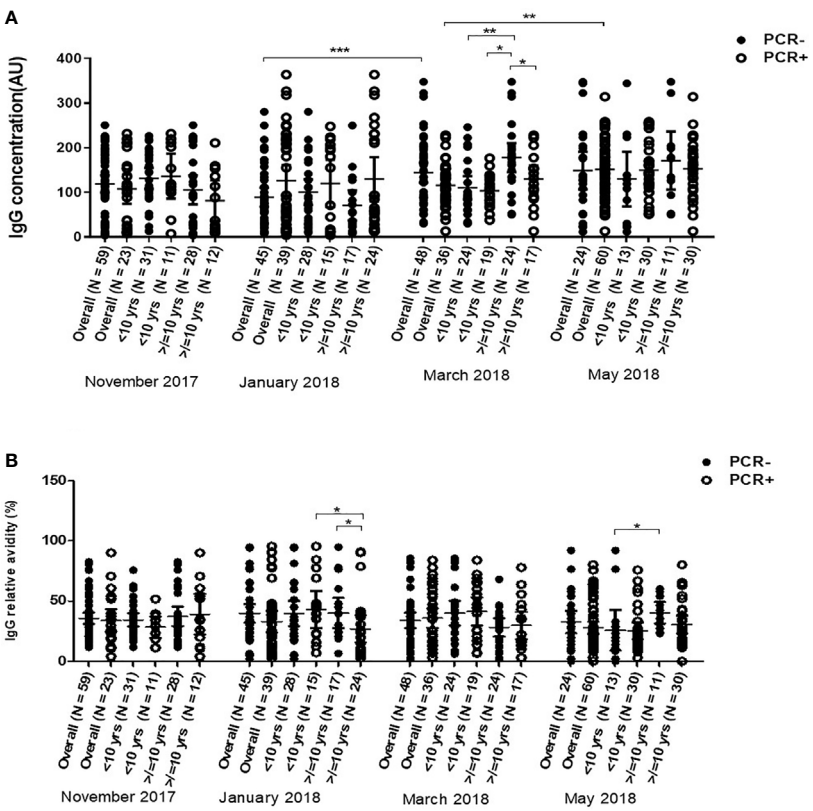

FIGURE 6 | Variations in of anti-Pfs230 IgG levels **(A)** and Relative avidities **(B)** in young children (<10 years) and older children (≥10 years) with (positive polymerase chain reaction test result) and without (negative-polymerase chain reaction test result) active *P. falciparum* infections over the dry season from November 2017 to May 2018. The number above each of the aligned dot plots represents the number of individuals in the group. The extent of statistically significant difference in measurements indicated by *, ** and *** represent P <0.05, P <0.01, and P <0.001 respectively.

TABLE 3 | Correlation between IgG level and avidity against Pfs230, Pfs48/45.

	Pfs48/45 IgG (level *vs* avidity)	Pfs230 IgG (level *vs* avidity)
Nov.2017	R = 0.0251, *P* = 0.7999	r = −0.5287, *P* <0.0001
Jan.2018	r = 0.4175, *P* <0.0001	r = −0.6911, *P* <0.0001
Mar.2018	r = −0.2177, *P* = 0.0360	r = −0.1824, *P* = 0.0800
May.2018	r = −0.2609, *P* = 0.0081	r = −0.2405, *P* = 0.0149

r, correlation coefficient; P, p-value; vs, versus.

parasite, relatively few reports have characterized the development of immunity against the sexual stages of *P. falciparum*. However, this knowledge can help improve the development of transmission-blocking vaccines to reduce malaria transmission. This study, for the first time, characterizes both quantity and quality of naturally acquired antibodies to both transmission-blocking vaccine candidates, Pfs230 and Pfs48/45 over the course of the dry season into the early rainy season.

This study was conducted at the end of the peak malaria season through to the beginning of the subsequent peak season, a period where there is a paucity of mosquito vectors (Amoah et al., 2018; Amoah et al., 2019a). The persistence of *P. falciparum* throughout the study period suggests that the possibly few mosquito vectors circulating during the off-peak season were able to sustain malaria transmission within the community. Carriage of *P. falciparum* can result in the development of anemia (Doolan et al., 2009; Magombedze et al., 2018; Epopa et al., 2019) however, most of the children in this study, despite harboring malaria had normal hemoglobin levels. This could however be because asymptomatic infections predominantly contain low parasite densities, which are less likely to result in anemia as compared with high parasite density infections (Doolan et al., 2009; Magombedze et al., 2018; Epopa et al., 2019).

IgG levels against both Pfs230 and Pfs48/45 were highest in May which is the beginning of the peak season. This observation was somewhat similar to our previous observation of higher anti-Pfs230 IgG levels in the peak season compared to the off-peak season and suggested an increased exposure to gametocytes compared to the preceding months assessed in this study (Amoah et al., 2019a). Although no gametocytes were identified by microscopy over the course of the study, the persistent replication of asexual parasites during the erythrocytic life cycle of the parasite is known to result in the production of low densities of gametocytes that are detectable by techniques with higher sensitivities than microscopy (Alano, 2007; Reuling et al., 2018). Prolonged exposure to submicroscopic gametocyte densities has previously been identified to induced antibody responses (Haldar & Mohandas, 2009; Amoah et al., 2019a; Muthui et al., 2020). A previous report from a similarly high malaria transmission setting of southern Ghana identified a higher prevalence of submicroscopic gametocytes densities during the off-peak season compared with the peak season (Ayanful-Torgby et al., 2018).

The trends in the relative avidity of IgG responses against Pfs48/45 antigen observed in both young and older children (<10 yrs or ≥10 yrs) were similar to the trend of asexual parasite prevalence estimated by microscopy. This is similar to observations made for the relative avidities of antibodies against asexual parasite antigens, which are low in high transmission settings due to frequent exposure to highly diverse parasite strains (Abagna et al., 2018). However, for most malaria infections, gametocyte densities are significantly lower than those of the asexual parasite (Alano, 2007; Reuling et al., 2018) and as such the lower numbers and diversity of gametocytes in the high transmission setting may be responsible for the inverse relationship between the relative avidity of antibody responses against sexual stage antigens relative to asexual stage antigens. The similarity of the pattern of the percentage seroprevalence and median parasite densities suggests that the level of antibodies produced was proportional to the parasitemia of infection.

Participants, especially older children (10–12 years old), with active infections generally showed a trend of lower antibody levels against both sexual stage antigens than those without active infection. This observation could be due to the concomitant large amounts of antibodies produced in response to recent past exposure to asexual and sexual stage parasites that are currently protecting against parasite infection. At the onset of the peak season (in May) the relative avidity of Pfs48/45 IgG in the young children (<10 years) with active infections were significantly higher than that of the older children (≥10 years) with and without active infection. A possible explanation for this could be the increase in exposure to low-density gametocytes in younger children relative to older children as gametocyte carriage has been suggested to be more prevalent in young children relative to the older population (Ouedraogo et al., 2007). The relative IgG avidity against Pfs230 at a time point close to peak malaria season was observed to be significantly higher in older children (≥10 years) than in younger children (<10 years), suggesting that age could have an impact on the avidity. This increase in IgG antibody avidity with the increase in age could be due to increased exposure to the same antigen and hence the tendency of IgG avidity to develop or have matured better in adulthood than in childhood as recently reported (Tassi Yunga et al., 2021). Lack of an association between the number of times a person had been infected with parasites during the follow-ups and antibody levels or avidities was not surprising as all microscopic infections determined contained only asexual parasites. Furthermore, most of the submicroscopic infections could contain only asexual forms of the parasite or gametocytes at thresholds not high enough to stimulate high levels of immune response.

The observed correlation between the level and relative avidity being positive for Pfs48/45 IgG but negative for that of Pfs230 IgG in November and January (early parts of the off-peak malaria season), could be suggestive of differences in decay kinetics associated with dynamic conformational flexibility (Huber, 1979; Amaral et al., 2017) of antibodies against both antigens, despite biological relatedness existing between both

antigens and a half-life of about three months having been determined for their antibodies. The negative correlation observed between IgG levels and avidities against both Pfs230 and Pfs48/45 in the second half of the study period, towards the peak malaria season, could be due to the effect of an increase in the frequency and persistence of high diversity infections on IgG responses (IgG avidities and levels) towards the peak season relative to the beginning as well as during the off-peak season (Sondo et al., 2020). Consistent infections have also been suggested to impair antibody affinity maturation in germinal centers and result in the production of antibodies with low avidity (Ssewanyana et al., 2017). An earlier study conducted in southern Ghana also reported an inverse relation between Pfs48/45 IgG levels and avidity (Amoah et al., 2019a).

Study Limitations

The quality of the antibodies against an antigen is determined by higher binding affinity as well as functionality. This study did not determine the functionality of the antibodies beyond determining the relative avidity index. Functional assays such as antibody-mediated complement lysis or Opsonic phagocytosis are important measures of antibody functionality. We could however not perform these assays due to limited funding. Furthermore, submicroscopic gametocyte densities, as well as the entomological inoculation rate during the period of the study, were also not determined. These could have provided additional measures of exposure rates as well as the risk of parasite infection due to gametocytes whose surface antigens were studied in the current study. However, studies have identified submicroscopic gametocyte densities in samples that were classified as negative for gametocytes by microscopy (Shekalaghe et al., 2007; Lamptey et al., 2018) as well as have identified a very low prevalence of mosquito vectors in the off-peak malaria season (Magombedze et al., 2018; Epopa et al., 2019).

CONCLUSION

IgG responses against Pfs48/45 and Pfs230 antigens were higher at the end of the off-peak season compared to the beginning. The relative avidities of IgG responses against Pfs230 and Pfs48/45 were inversely correlated to their IgG levels from the middle to the end of the off-peak season.

DATA AVAILABILITY STATEMENT

The datasets used and/or analyzed during the current study are included in the manuscript or **Supplementary Material**.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by The Institutional Review Board (IRB) of the Noguchi Memorial Institute for Medical Research (NMIMR). Written informed consent to participate in this study was provided by the participant's legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

LA designed the study. LA, FA, and FB performed the statistical analysis. ES and FB performed the experiments. EO and ES collected the samples. LA, D-OY, FA, EO, ES, and FB wrote and revised the final manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

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MINI REVIEW
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Identification of Novel Malaria Transmission-Blocking Vaccine Candidates

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Control measures have significantly reduced malaria morbidity and mortality in the last two decades; however, the downward trends have stalled and have become complicated by the emergence of COVID-19. Significant efforts have been made to develop malaria vaccines, but currently only the RTS,S/AS01 vaccine against *Plasmodium falciparum* has been recommended by the WHO, for widespread use among children in sub-Saharan Africa. The efficacy of RTS,S/AS01 is modest, and therefore the development of more efficacious vaccines is still needed. In addition, the development of transmission-blocking vaccines (TBVs) to reduce the parasite transmission from humans to mosquitoes is required toward the goal of malaria elimination. Few TBVs have reached clinical development, and challenges include low immunogenicity or high reactogenicity in humans. Therefore, novel approaches to accelerate TBV research and development are urgently needed, especially novel TBV candidate discovery. In this mini review we summarize the progress in TBV research and development, novel TBV candidate discovery, and discuss how to accelerate novel TBV candidate discovery.

Keywords: immuno-profiling, malaria, *Plasmodium*, reverse vaccinology, transmission-blocking vaccine (TBV), wheat germ cell-free system (WGCFS)

INTRODUCTION

Malaria continues to be responsible for a substantial global health burden, with 409,000 malarial deaths reported in 2019 (WHO, 2020). From 2000 to 2015, malaria morbidity and mortality were significantly reduced; however, the decreasing trend stalled between 2015 and 2019 and was further complicated by the emergence of COVID-19 (Wang et al., 2020; WHO, 2020). Therefore, the

Abbreviations: AnAPN1, anopheline alanyl aminopeptidase N 1; BDES, baculovirus dual expression system; *E.*, *Escherichia*; EPA, ExoProtein A from *Pseudomonas aeruginosa*; HAP2/GCS1, Hapless 2/Generative Cell Specific 1; IFA, indirect immunofluorescence assay; MiGS, microgamete surface protein; *P.*, *Plasmodium*; PH, pleckstrin homology; PSOP, putative secreted ookinete protein; SMFA, standard membrane feeding assay; TBA, transmission-blocking activity; TBV, transmission-blocking vaccine; TRA, transmission-reducing activity; WGCFS, wheat germ cell-free system; WHO, The World Health Organization.

control and eventual eradication of this disease relies on the development of a highly effective malaria vaccine.

Malaria vaccines can be categorized into three groups, each targeting distinct parasite developmental stages: pre-erythrocytic (sporozoite and liver), asexual erythrocytic, and sexual transmission stages. The renewed Malaria Vaccine Technology Roadmap proposes two main objectives by 2030 for the development of new malaria vaccines targeting both *Plasmodium falciparum* and *Plasmodium vivax*: i) vaccines with protective efficacy of at least 75% against clinical malaria, and ii) vaccines that reduce transmission of the parasite (Group, 2013; Moorthy et al., 2013). A leading malaria vaccine RTS,S/AS01 was the first malaria vaccine to enter Phase III clinical trials and shows modest efficacy against clinical falciparum malaria (RTS, 2015) with short durability (White et al., 2015). It is currently being evaluated in a large pilot implementation program in Ghana, Kenya, and Malawi since 2019 (Adepoju, 2019). The vaccine reduced severe malaria by about 30% in the first 2 years of the program (Vogel, 2021). Based on this, the World Health Organization (WHO) is now recommending widespread use of the RTS,S/AS01 malaria vaccine among children in sub-Saharan Africa and in other regions with moderate to high *P. falciparum* malaria transmission (Vogel, 2021).

Since the RTS,S/AS01 vaccine efficacy is modest, the development of more efficacious vaccines is still needed. A number of second-generation malaria vaccines are in clinical trials, such as R21/Matrix-M (Datoo et al., 2021). However, the above mentioned two malaria vaccines are classified as pre-erythrocytic stage vaccines. Therefore, the development of erythrocytic stage vaccines to reduce morbidity and mortality, and transmission-blocking vaccines (TBVs) to reduce parasite transmission from humans to mosquitoes, are required to reach the Roadmap goals.

MALARIA TRANSMISSION-BLOCKING VACCINES (TBVS)

The principle of malaria TBVs is that antibodies against antigen(s) expressed on the sexual stages of the malaria parasite - gametocyte/gamete/zygote/ookinete - reduce the numbers of oocysts in mosquito vectors when fed with gametocytes (Huff et al., 1958; Carter and Chen, 1976; Gwadz, 1976). The advantages of TBVs are summarized as follows (Tsuboi et al., 2003; Miura et al., 2019; Duffy, 2021): i) TBV candidates tend to be less polymorphic than blood- or pre-erythrocytic-stage antigens, presumably due to lower immune pressure driving evolutionary diversity; ii) the absolute number of parasites targeted by TBVs is small, usually <10-100 oocysts per mosquito in nature, and represent a biological bottleneck in the malaria parasite lifecycle; and iii) TBVs might help to prevent the spread of emerging drug-resistant parasites (Dondorp et al., 2009; Balikagala et al., 2021) and future vaccine-escape mutants.

Target antigens include proteins expressed on the surface of gametocytes/gametes/zygotes/ookinetes; such as the characterized proteins P230, P48/45, P28, and P25 (Carter and Kaushal, 1984; Kumar and Carter, 1985; Vermeulen et al., 1985). To initiate vaccine research, the antigens in human malaria parasites were identified in the pre-genomic era; namely, Pfs25 (Kaslow et al., 1988; Kaslow et al., 1994), Pfs28 (Duffy and Kaslow, 1997), Pfs48/45 (Kocken et al., 1993; Outchkourov et al., 2008), and Pfs230 (Williamson et al., 1993; Williamson et al., 1995) from *P. falciparum*; and their orthologs in *P. vivax*, Pvs25 and Pvs28 (Tsuboi et al., 1998; Hisaeda et al., 2000). Soon after whole genome information became accessible, Pvs48/45 (Arevalo-Herrera et al., 2015; Tachibana et al., 2015) and Pvs230 (Tachibana et al., 2012) were also characterized as TBV candidates (**Table 1**).

Researchers have faced a number of difficulties to express TBV antigens with native conformations (Miura et al., 2019), using a variety of protein expression systems (Patel and Tolia, 2021). Antibodies raised against individual antigens needed to be tested in an *ex vivo* efficacy assay; specifically, the standard membrane feeding assay (SMFA) wherein laboratory-reared *Anopheles* mosquitoes are fed on *in vitro* cultured *P. falciparum* gametocytes along with test antisera or purified antibodies, and counts of midgut wall oocysts as a measure of the degree of transmission-blocking activity (Miura et al., 2013a).

TBV DEVELOPMENT EFFORTS TO DATE

After decades of efforts, the most advanced *P. falciparum* TBV antigens in the clinical pipeline remain the first identified antigens: Pfs25 expressed on the surface of zygotes/ookinetes in the mosquito and classified as a post-fertilization antigen, and Pfs48/45 and Pfs230 expressed on the surface of blood-circulating gametocytes and gametes in the mosquito and classified as pre-fertilization antigens. In addition, a mosquito midgut protein, anopheline alanyl aminopeptidase N 1 (AnAPN1) (Armistead et al., 2014), is under development as a TBV candidate in pre-clinical developmental studies (Bender et al., 2021) (**Table 1**, **Figure 1**). As transmission-blocking immunity is mostly antibody-mediated (de Jong et al., 2020), TBV development efforts focus on inducing potent antibodies that are sustained at effective transmission-blocking levels for at least one transmission season. Based on these requirements, extensive efforts towards the clinical development of *P. falciparum* TBVs continue to date. Recently, phase 1 trials of *P. falciparum* TBV based upon Pfs25/Alhydrogel (Alum) have been reported. These studies used Pfs25-EPA: Pfs25 conjugated with a recombinant detoxified ExoProtein A from *Pseudomonas aeruginosa* (EPA), formulated with Alum, and tested in adults in the USA (Talaat et al., 2016) and Mali (Sagara et al., 2018). The vaccine was generally well-tolerated; however, the functional activity of the anti-Pfs25 antibodies induced were modest, and antibody titers decreased rapidly.

To improve functional immunogenicity and durability, the same group performed a phase 1 trial of the pre-fertilization TBV antigen Pfs230 alone or in combination with Pfs25 in USA adults. Pfs25-EPA/Alum and Pfs230D1M [amino acid 542-736 (MacDonald et al., 2016)]-EPA/Alum induced similar serum functional activity in mice, but Pfs230D1M-EPA induced significantly greater activity in rhesus monkeys. In USA adults,

TABLE 1 | Discovery of malaria transmission-blocking vaccine antigens with publication years[a].

Antigen[b]	Year[c]	Target parasite[d]	Developmental stage[e]	Discovery[f]	Expression system[g]	Reference
Pre-Genomic Era						
Pfs25	1988	*Pf*	Zygote/ookinete	Gene	–	(Kaslow et al., 1988)
Pfs25	1994	*Pf*	Zygote/ookinete	TRA	Yeast	(Kaslow et al., 1994)
Pfs28	1997	*Pf*	Zygote/ookinete	Gene/TRA	Yeast	(Duffy and Kaslow, 1997)
Pfs48/45	1993	*Pf*	Gametocyte/gamete	Gene	–	(Kocken et al., 1993)
Pfs48/45	2008	*Pf*	Gametocyte/gamete	TRA	Bacteria	(Outchkourov et al., 2008)
Pfs230	1993	*Pf*	Gametocyte/gamete	Gene	–	(Williamson et al., 1993)
Pfs230	1995	*Pf*	Gametocyte/gamete	TRA	Bacteria	(Williamson et al., 1995)
Pvs25 & Pvs28	1998	*Pv*	Zygote/ookinete	Gene	–	(Tsuboi et al., 1998)
Pvs25 & Pvs28	2000	*Pv*	Zygote/ookinete	TRA	Yeast	(Hisaeda et al., 2000)
Post-Genomic Era						
HAP2/GCS1	2008	*Pb*	Gamete	Gene	–	(Hirai et al., 2008; Liu et al., 2008)
HAP2/GCS1	2009	*Pb*	Gamete	TRA	Bacteria	(Blagborough and Sinden, 2009)
HAP2/GCS1	2013	*Pf*	Gamete	TRA	WGCFS	(Miura et al., 2013b)
HAP2/GCS1	2017	*Pb, Pf*	Gamete	TRA	Peptide	(Angrisano et al., 2017)
HAP2/GCS1	2020	*Pv*	Gamete	TRA	Baculovirus	(Qiu et al., 2020)
Pvs230	2012	*Pv*	Gametocyte/gamete	TRA	DNA	(Tachibana et al., 2012)
Pvs48/45, Pvs47	2015	*Pv*	Gametocyte/gamete	TRA	DNA, Bacteria	(Arevalo-Herrera et al., 2015; Tachibana et al., 2015)
Pfs47	2010	*Pf*	Gametocyte/gamete	Gene	–	(van Schaijk et al., 2006)
Pfs47	2018	*Pf*	Gametocyte/gamete	TRA	Bacteria	(Canepa et al., 2018)
AnAPN1	2014	*Pf, Pv*	*Anopheles* midgut	Gene/TRA	*Drosophila* S2	(Armistead et al., 2014)
PbPSOP12	2015	*Pb*	Gamete - ookinete	TRA	BDES	(Sala et al., 2015)
PbPH	2016	*Pb*	Gamete - ookinete	TRA	Bacteria	(Kou et al., 2016)
PbPSOP7, 25 & 26	2016	*Pb*	Ookinete	TRA	Bacteria	(Zheng et al., 2016)
PbPSOP25	2017					(Zheng et al., 2017)
Pb51	2017	*Pb*	Gametocyte - ookinete	TRA	Bacteria	(Wang et al., 2017)
Pbg37	2018	*Pb*	Gametocyte - zygote	TRA	Bacteria	(Liu et al., 2018)
PyMiGS	2018	*Py*	Gametocyte/gamete	TRA	WGCFS	(Tachibana et al., 2018a; Tachibana et al., 2018b; Tachibana et al., 2020)
	2020					
Pb22	2021	*Pb*	Gamete - ookinete	TRA	Bacteria	(Liu et al., 2021)

[a]*Summary of the TBV antigen discovery efforts in which significant TRA has been confirmed.*
[b]*Antigen, abbreviated names of TBV antigens.*
[c]*Year, year of publication.*
[d]*Target parasite, Pf, Plasmodium falciparum; Pv, P. vivax; Pb, P. berghei; Py, P. yoelii.*
[e]*Developmental stage, parasite developmental stage(s) in which target the antigen is expressed.*
[f]*Discovery, Gene, target gene discovered; TRA, antigens specific antibodies with confirmed transmission reducing/blocking activity identified..*
[g]*Expression system, indicates the platform used to express the antigen as either in yeast cells, bacteria, wheat germ cell-free system (WGCFS), Drosophila S2 cells, baculovirus vectored protein expression system or was a synthetic peptide (Peptide). Alternatively, DNA vaccine used as the antigen (DNA). BDES, indicates target antigen was expressed in baculovirus dual expression system.*

two vaccine doses induced functional activity in Pfs230D1M-EPA/Alum volunteers, but no significant activity in Pfs25-EPA vaccine recipients, and combination with Pfs25-EPA did not increase functional activity over Pfs230D1M-EPA alone. The research group concluded that the functional activity of Pfs230D1M-EPA is significantly superior to that of Pfs25-EPA (Healy et al., 2021). For more information about the clinical development of these falciparum TBVs, please refer to two recent review articles (Miura et al., 2019; Duffy, 2021). In addition to the above TBV development efforts, novel TBV candidate discovery is required to accelerate the success in TBV development.

POST-GENOME NOVEL TBV CANDIDATE DISCOVERY

The goal of identifying new vaccine candidates for both *P. falciparum* and *P. vivax* is aided by whole genome information accessible since 2003 at the malaria genome database (PlasmoDB). The database has been useful to identify vaccine candidates from asexual-blood (Kanoi et al., 2021) and pre-erythrocytic (Bettencourt, 2020) stages. However, the rational selection and prioritization of TBV candidates from the database has yet to be fully explored (Miura et al., 2019). Extensive proteome and transcriptome data from sexual-stage malaria parasites is now available (Lasonder et al., 2016; Meerstein-Kessel et al., 2018) to inform *in silico* TBV candidate discovery. In the following sections we summarize the recent achievements for the discovery of the activities and candidate antigens discovered in the post-genomic era (**Table 1**, **Figure 1**).

RODENT MALARIA MODELS FOR NOVEL TBV CANDIDATE DISCOVERY

Most of the TBV candidates investigated to date have orthologs in rodent malaria parasites, and thus the rodent malaria models

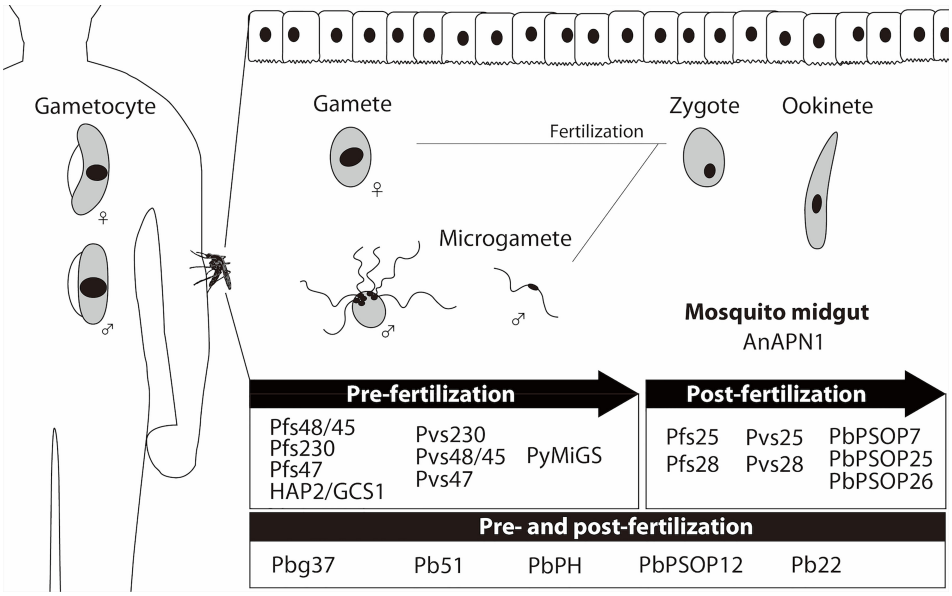

FIGURE 1 | Expression of malaria transmission-blocking vaccine (TBV) target antigens. Sexual developmental stages of malaria parasites in humans (gametocytes) and mosquitoes (gametes, zygotes, and ookinetes) are schematically presented. The TBV candidate antigens (**Table 1**) are categorized as pre-fertilization antigens (mainly expressed in the sexual stages of parasites before fertilization), and post-fertilization antigens (mainly expressed in the sexual stages of parasites after fertilization). Mosquito midgut antigen, AnAPN1, is also presented as a TBV candidate.

are useful for the discovery and characterization of novel TBV candidates. In the last decade several potential TBV candidates have been identified using rodent malaria models. The general strategy of these studies is to select candidate genes from the PlasmoDB according to the following criteria: i) genes must be specifically expressed in sexual-stages; ii) they must share orthologs with human parasites, in particular *P. falciparum* and *P. vivax*; and iii) the presence of a predicted signal peptide with/without transmembrane domain(s) or a GPI-anchor, indicating possible protein export and exposure to inhibitory antibodies. Candidate TBV genes are then expressed in one or more recombinant protein expression systems, followed by immunization of mice. To test efficacy, immunized mice are infected with rodent malaria parasites and then mosquitoes are fed directly on these mice; termed a direct feeding assay. The transmission-blocking activity (TBA) is expressed as a percent reduction of the prevalence of infected mosquitoes; and transmission-reducing activity (TRA) is expressed as a percent reduction of oocyst density.

The majority of such studies were conducted with *P. berghei* rodent parasites because of the ease for genetic manipulation, such as the knockout of candidate genes for functional characterization of novel TBV candidates. Most such activities are listed in **Table 1**, classified in the post-genomic era, and following are descriptions of examples of post-genomic studies.

As the first examples, a group actively working on novel TBV candidate discovery using the *P. berghei* model identified a conserved *P. berghei* protein, PbPH, containing a pleckstrin homology (PH) domain. By indirect immunofluorescence assay (IFA) PbPH localized on the surface of gametes/zygotes/ookinetes. Mice were immunized with recombinant PbPH expressed in *E. coli* and mosquitoes fed on the immunized mice showed a 48% TRA (Kou et al., 2016). Similarly, the same group selected *P. berghei* ookinete-stage proteins, Putative Secreted Ookinete Protein (PbPSOP25), PbPSOP26, and PbPSOP7, for evaluation of their transmission-blocking potentials. Antisera against these bacterially expressed partial recombinant proteins recognized the ookinete surface. Mosquitoes fed on immunized mice showed significant TRAs (60% to 71%) (Zheng et al., 2016). Mice immunized with full-length recombinant PSOP25 expressed in *E. coli* and those receiving passive transfer of an anti-rPSOP25 mAb showed significant TRAs by 66% and 63%, respectively (Zheng et al., 2017).

The conserved *Plasmodium* gene, Pb51, was identified in *P. berghei* through PlasmoDB using gene expression and protein localization criteria. A partial domain of Pb51 was expressed in *E. coli* and mice were immunized. By IFA Pb51 was expressed in schizonts/gametocytes/ookinetes of *P. berghei*. Mice immunized with the recombinant Pb51 showed 55% TRA in direct feeding assays (Wang et al., 2017). Using a similar approach, the same group characterized a protein of 37 kDa preferentially expressed in gametocytes in *P. berghei* (Pbg37). A recombinant Pbg37 (rPbg37) was expressed in bacteria and antibody was generated in mice. IFA showed surface expression of Pbg37 on gametes/zygotes. The rPbg37-immunized mice had a significant TRA (49%) (Liu et al., 2018). Similarly, a gamete/ookinete surface protein of *P. berghei*, Pb22, was identified and recombinant Pb22 was expressed in *E. coli*. The Pb22-immunised mice had a significant TRA (93.5-99.6%) (Liu et al., 2021).

The *P. berghei* ookinete-stage protein, PbPSOP12, was identified based upon annotation as a putative secreted protein and then expressed using the baculovirus dual expression system (BDES). Mouse antibodies against BDES-PbPSOP12 recognized the surface of gametes/ookinetes. Immunization of mice with BDES-PbPSOP12 conferred modest TRA (53%) (Sala et al., 2015).

Our lab has accumulated a number of experiences using the *Plasmodium yoelii* rodent malaria parasite as a suitable model for TBV study; such as the identification of Pfs25 and Pfs28 orthologs in *P. yoelii*, Pys25 and Pys28 (Tsuboi et al., 1997a; Tsuboi et al., 1997b; Tsuboi et al., 1997c). Recently we identified a novel TBV candidate, *P. yoelii* microgamete surface protein (PyMiGS), using a similar approach as mentioned above for the *P. berghei* studies. PyMiGS is a protein expressed in the osmiophilic body of male gametocytes of *P. yoelii* and is translocated to the surface of microgametes. Potent TRA (>99%) was observed in mosquitoes fed on mice passively immunized with antibodies against recombinant full-length PyMiGS expressed using a wheat germ cell-free protein expression system (WGCFS) (Tachibana et al., 2018a). Mice actively immunized with the recombinant full-length PyMiGS conferred >99% TRA using direct mosquito feeding (Tachibana et al., 2018b), and the major epitopes for transmission-blocking antibodies were within the C-terminal region of PyMiGS (Tachibana et al., 2020).

Although the *P. berghei* and *P. yoelii* rodent malaria models are useful to identify novel TBV candidates, results between the models may differ. For example, when we characterized the phenotype of a PyMiGS gene deletion mutant (ΔPyMiGS), the ookinete formation efficiency of ΔPyMiGS was significantly impaired (Tachibana et al., 2018a). Contrary, ookinete formation of the gene deletion mutant of the *P. berghei* ortholog of PyMiGS (PBANKA_1449000) was not impaired (Kehrer et al., 2016). Accordingly, although the usefulness of the rodent models is clear, careful consideration is also required.

Candidates identified in the rodent malaria studies should be evaluated with *P. falciparum* orthologs. For example, a conserved male gamete sterility gene, HAP2/GCS1 (Hapless 2/Generative Cell Specific 1), was initially identified as an essential protein for the fusion of male and female gametes of *P. berghei* (Hirai et al., 2008; Liu et al., 2008). Genetic disruption of the *hap2* locus revealed that parasite fertilization is inhibited, and anti-PbHAP2 sera showed TRA by up to 81% (Blagborough and Sinden, 2009). Mosquitoes fed on mice immunized with PbHAP2 cd loop peptide showed 59% TRA in *P. berghei* and the corresponding TRA in *P. falciparum* was 76% (Angrisano et al., 2017). We also demonstrated strong transmission-blocking activity of mouse antibody against recombinant *P. falciparum* HAP2 protein and concluded the antigen to be a novel TBV candidate (Miura et al., 2013b). Recently, recombinant *P. vivax* HAP2 was expressed in a baculovirus expression system, and rabbit antibody induced significant TRA (40% to 90%) against *P. vivax* field isolates in *Anopheles dirus* (Qiu et al., 2020).

The gametocyte/gamete protein P47 is another example of experimental system-specific differences. When the *p47* gene was disrupted, a strong reduction of female fertility was observed in *P. berghei* (van Dijk et al., 2010), but not in *P. falciparum* (van Schaijk et al., 2006), and anti-Pfs47 mAbs showed no efficacy in *P. falciparum* SMFA (van Schaijk et al., 2006). Similarly, mAbs and polyclonal antibodies against a full-length recombinant Pfs47 protein did not show efficacy in SMFA. However, antibodies against a part of domain 2 in Pfs47 did demonstrate significant TRA (Canepa et al., 2018). Further characterization revealed that when mice were immunized with the full-length protein, almost no antibody was induced against the critical domain 2. Therefore, it is possible that other potential TBV candidates were overlooked in previous studies (Miura et al., 2019); and improvement of antigen design and vaccine formulations with existing TBV candidates, and expansion of the repertoire of novel TBV candidates, are necessary to accelerate TBV development.

NOVEL TBV CANDIDATE DISCOVERY DIRECTLY USING HUMAN MALARIA PARASITES

In *P. falciparum* only two studies on genome-wide novel TBV candidate discovery have been reported to date. One is a reverse vaccinology approach by Nikolaeva et al. (Nikolaeva et al., 2020). They identified a panel of potential TBV candidate genes from PlasmoDB by selecting with a sexual-stage specific expression profile. After a logical *in-silico* process to narrow down the candidate list, they expressed 21 recombinant proteins using a human embryonic kidney cell (HEK293) expression system. Twelve proteins were successfully expressed, and mouse antibodies against the recombinant proteins were tested by SMFA. However, none of the novel TBV candidates showed TRA. It is possible that the heterologous human cell expression system resulted in aberrant glycosylation patterns compared with *Plasmodium*, which has a minimal glycosylation machinery, and the resulting antibodies did not recognize native *Plasmodium* protein (Kanoi et al., 2021).

The other is a larger-scale trial of immuno-profiling of naturally occurring antibody-mediated TRA (Stone et al., 2018). Bioinformatically selected 315 proteins were expressed using an *E. coli* cell-free system, and correctly-folded well-characterized recombinant Pfs48/45 and Pfs230 proteins were used as positive controls. They assessed antibody responses in 648 African plasma samples with TRA measured by SMFA, and those with high (≥ 90%, n= 22) or low (< 10%, n=254) TRA were used for the immuno-profiling. Forty-three out of 315 proteins in addition to Pfs230 and Pfs48/45 had significantly higher antibody levels in plasmas with high TRA. After additional consideration on the protein expression levels in gametocytes, and the presence of a signal peptide or a transmembrane domain, 13 out of the 43 proteins were selected as possible TBV candidates. Although the strategy of this work is convincing, to date they have not validated whether any of the 13 novel TBV candidates could induce transmission-blocking antibodies in immunized animals. In addition, since the reacted human

antibodies were likely to recognize only linear epitopes of the tested antigens, because the proteins were expressed in *E. coli*, this work may have missed promising candidates which have conformational TRA epitopes/antigens (Miura et al., 2019). Finally, the approach might not identify TBV candidates whose protein expression is solely in the mosquito and not in gametocytes.

Additional gametocyte-specific gene discovery efforts have been published (Ikadai et al., 2013; Chawla et al., 2021; Muthui et al., 2021); although antigen expression, immunization, and TRA assessment of the antibodies are not completed.

KEY MESSAGES TO THE NOVEL TBV CANDIDATE DISCOVERY

The clinical development of *P. falciparum* TBV have advanced to Phase 2 clinical trials (Duffy, 2021). However, those efforts have focused only on the leading candidates - Pfs25, Pfs230, and Pfs48/45 - which were identified in the pre-genome era (Miura et al., 2019). To accelerate TBV research and development in the post-genome era, genome-wide discovery of novel TBV candidates by both immuno-profiling and reverse vaccinology approaches are essential. A key message learned from the pioneering post-genome TBV candidate discovery approaches is that it is crucial to select an expression system with the capability of producing large numbers of correctly-folded malaria recombinant proteins, and without artificial glycosylation. We have been using the WGCFS to express a number of high-quality recombinant proteins of both *P. falciparum* and *P. vivax*; and to produce comprehensive genome-wide protein libraries useful for novel malaria vaccine and sero-marker candidate discovery projects (Morita et al., 2017; Kanoi et al., 2018; Longley et al., 2020; Kanoi et al., 2021). Therefore, following genome-wide gametocyte stage protein expression by WGCFS, these proteins can then be used in immuno-profiling approaches using human plasma with known TRA, to identify novel transmission-blocking antigens (Ntege et al., 2017; Miura et al., 2019; Kanoi et al., 2021). To this end it is also essential to obtain well-characterized plasma samples from infected individuals who carry transmission-reducing antibodies.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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REVIEW
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Stressed Out About *Plasmodium falciparum* Gametocytogenesis

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Blocking malaria transmission is critical to malaria control programs but remains a major challenge especially in endemic regions with high levels of asymptomatic infections. New strategies targeting the transmissible sexual stages of the parasite, called gametocytes, are needed. This review focuses on *P. falciparum* gametocytogenesis *in vivo* and *in vitro*. Highlighting advances made elucidating genes required for gametocyte production and identifying key questions that remain unanswered such as the factors and regulatory mechanisms that contribute to gametocyte induction, and the mechanism of sequestration. Tools available to begin to address these issues are also described to facilitate advances in our understanding of this important stage of the life cycle.

Keywords: malaria, transmission, sexual differentiation, gametocyte, developmental regulation, bone marrow

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INTRODUCTION

Nearly half of the world's population is still at risk of malaria (WHO, 2020). Through the 1980s and 1990s, the spread of *P. falciparum* parasites resistant to chloroquine, the mainstay of treatment since the 1950s, across Africa increased the annual malaria mortality rate above one million (Nuwaha, 2001). The introduction of artemisinin combination therapy in 2004 coupled with increased distribution of insecticide-treated bed nets and enhanced vector control efforts reduced the prevalence of malaria 27.9% and mortality declined 42.5% by 2017 (Weiss et al., 2019). Intense efforts to prevent, detect and treat the disease have allowed several countries to successfully eliminate malaria. Algeria and Argentina in 2019 and China and El Salvador in 2021 have been certified as malaria-free and globally, a total of 40 countries and territories have now been declared malaria-free (WHO, 2021). As more highly endemic countries move toward malaria elimination, comprehensive approaches to accurately define the human malaria reservoir and the impact of both symptomatic and asymptomatic infections are required to effectively interrupt transmission.

Malaria infection begins when a *Plasmodium*-infected *Anopheles* mosquito introduces saliva containing parasites, called sporozoites, during a blood meal. Sporozoites travel through the bloodstream and invade liver cells where they replicate asexually producing 10,000-30,000 new merozoites that are released into the bloodstream when the liver cell ruptures. Merozoites invade red blood cells (RBCs) and either replicate asexually producing 16-32 new merozoites in 48 hours or differentiate into a single male or female gametocyte (**Figure 1**). The new merozoites are released by RBC rupture and the cycle repeats until it is controlled by the immune response or drug treatment. In contrast, the gametocytes produced during each cycle are terminally differentiated and die unless they are taken up in a blood meal by a mosquito. Once in the mosquito midgut, gametocytes are stimulated

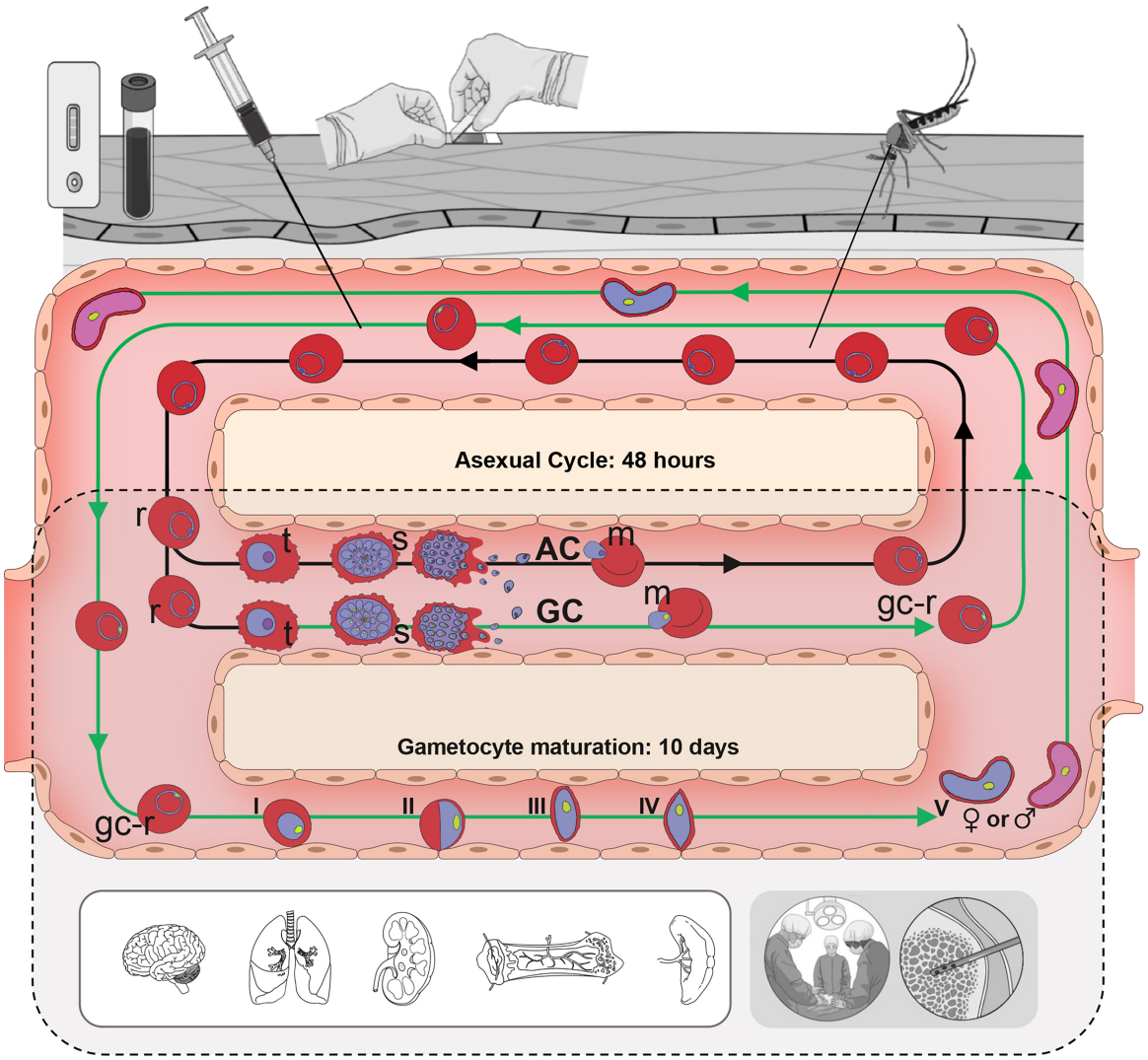

FIGURE 1 | Human *Plasmodium falciparum* life cycle. In the bloodstream, asexually-committed (AC) merozoites (m, purple nucleus) invade RBCs and begin the asexual replication cycle (black arrows), passing through ring (r), trophozoite (t) and schizont (s) stages. During this development some parasites become gametocyte-committed (GC, green arrows) producing gametocyte-committed merozoites (green nucleus). Gc-merozoites invade RBCs and begin differentiating through 5 morphologically distinct stages (I-V) over the next 10 days to become mature stage V gametocytes. *P. falciparum* trophozoites, schizonts and immature stage I–IV gametocytes sequester and cannot be detected in peripheral blood samples (grey shading). Therefore, a blood sample obtained by a syringe, finger prick or mosquito bite only contains ac- and gc-rings and stage V gametocytes, while tissue samples are needed to collect all developmental stages.

to undergo gametogenesis and fertilization resulting over the next 2 weeks in the production of tens of thousands of sporozoites that can be released with the saliva to initiate another infection.

This basic life cycle was described 121 years ago by Battista Grassi (Grassi, 1901) and at most steps a change in environment, either switching from host bloodstream to mosquito midgut or mosquito salivary gland to host liver, triggers the entire parasite population to begin a specific developmental program. The only exception to this for most species is the decision between asexual and sexual development during the intraerythrocytic cycle. Both stages develop in erythrocytes in the human bloodstream but have different outcomes. Asexual replication is required to maintain and increase parasite levels in the human host, while terminally differentiated gametocytes are required for transmission. If all the intraerythrocytic parasites commit to sexual differentiation at once, there would be no further increase in parasitemia and the infection would be over except for maturing gametocytes that survive for several days. The gametocyte circulation time would provide a small window for transmission *via* a mosquito to another person. Instead, the parasite has evolved so that only a subpopulation of parasites commits to sexual differentiation each cycle. This allows the host parasitemia to be maintained and provides a continuous supply of gametocytes that are available whenever a mosquito takes a blood meal.

GAMETOCYTE DEVELOPMENT

The time course of gametocyte production varies in different Plasmodium species. In *P. falciparum*, which is the focus of this review and responsible for the most virulent human malaria, gametocyte maturation within the RBC takes 10-12 days and has 5 distinct morphological stages (I-V) that can be observed in *in vitro* culture (Hawking et al., 1971). *In vivo* the time course is the same, but immature *P. falciparum* gametocytes (stage I-IV) are sequestered, primarily in the bone marrow and spleen (Thomson, 1914). The absence of immature gametocytes in peripheral blood samples complicates the evaluation of gametocytogenesis in the field. Once mature, stage V gametocytes are released back to circulation they survive a median of 6 days (Eichner et al., 2001). It is only during this time gametocytes can be picked up by a mosquito or in a blood sample for analysis using Giemsa-stained blood smears or reverse transcriptase-quantitative polymerase chain reaction (RT-qPCR). Gametocytes are not killed by current commonly used antimalarials and therefore, even after successful treatment, the long maturation and survival time of gametocytes allows continued transmission for over a week. At least one male and one female gametocyte must be taken up in a blood meal (~1 μl) for fertilization and further sporogonic development suggesting a theoretical limit of 2 gametocytes/μl or 0.00004% gametocytemia, which is below the sensitivity of a Giemsa stained smear. This means that the asexual parasitemia had to be at or above this parasitemia 10-12 days prior (**Figure 2**). Using the average sexual conversion rate observed in culture for *P. falciparum* strain NF54 of ~10% an asexual parasitemia of 0.0004% or 20 parasites/μl could produce enough mature gametocytes to spread the disease 10-12 days later. In malaria-endemic regions, asexual parasitemias in both asymptomatic and symptomatic carriers are often well above 20 parasites/μl suggesting that with a 10% conversion rate all these individuals could be important infectious reservoirs.

THE INFECTIOUS RESERVOIR

While young children < 5 years old report the highest rates of symptomatic *P. falciparum* cases, many people experience asymptomatic infection, including older children and adults (Doolan et al., 2009; Lindblade et al., 2013; Rodriguez-Barraquer et al., 2018). Although there is no standard definition of asymptomatic infection, it generally means a person with detectable circulating parasites but no fever or other acute symptoms (Lindblade et al., 2013). The increasing use of sensitive molecular diagnostics such as PCR revealed the high prevalence of asymptomatic infections and their potential as an infectious reservoir that could threaten malaria elimination in endemic areas (Lindblade et al., 2013; Goncalves et al., 2017). In contrast to individuals with acute symptomatic infections, those with asymptomatic infections do not seek treatment and are unlikely to be detected or treated (Nassir et al., 2005). Consequently, asymptomatic infections can be of longer duration and allow more time for gametocyte production (WWARN-Gametocyte-Study-Group, 2016). A recent multi-level logistic regression model that controlled for parasite density and mosquito abundance found that asymptomatic infections had 2.66-fold greater odds of malaria transmission to mosquitoes than symptomatic infections (Sumner et al., 2021). In a large study in Burkina Faso and Kenya, children, who harbor the highest parasite and gametocyte densities, are more infectious to mosquitoes than adults, but adults (> 15 yrs. old) have more exposure to mosquitoes, resulting in a greater contribution to falciparum infection in mosquitoes (Goncalves et al., 2017). Another concern is that asymptomatic individuals don't know they are infected and can pass border screening and become a source of imported malaria under favorable conditions. This imported malaria is considered a serious challenge to malaria elimination (Lin et al., 2014; Domínguez García et al., 2019; Mischlinger et al., 2020) and has been the source of malaria resurgences in malaria-free countries (Nasir et al., 2020).

FACTORS ASSOCIATED WITH GAMETOCYTE CARRIAGE AND *IN VIVO* GAMETOCYTOGENESIS

Although recent fieldwork has revealed the importance of asymptomatic infectious reservoirs, historically the factors associated with *P. falciparum* gametocyte carriage *in vivo* have been evaluated in symptomatic infections, which are much easier to monitor. A recent meta-analysis of clinical trials conducted on three continents, Asia, Africa, and South America, showed that, on average, gametocytes were detectable in blood smears in 12.1% of nearly 50,000 patients (WWARN-Gametocyte-Study-Group, 2016). Further analysis indicated that the prevalence of gametocytemia before treatment was negatively associated with age, parasitemia, fever (axillary temperature >37.5°C or reporting febrile symptoms), and hemoglobin concentration. This finding is consistent with previous reports of associations between stage V gametocyte prevalence and in low hematocrit (Drakeley et al., 1999; Stepniewska et al., 2008). A history of illness >2 days (Price et al., 1999; Sowunmi et al., 2004) has also been linked to stage V gametocyte carriage, but whether these factors are causal is difficult to determine from these studies. As mentioned before, stage V gametocytes collected in a blood sample began sexual differentiation 10-16 days before so it is possible that the low hemoglobin, and > 2-day history of fever could be associated with a longer infection (Garnham, 1931). Recent work monitoring immature and mature gametocytes in bone marrow aspirates and peripheral blood samples from anemic children also found a significant correlation between low hemoglobin levels, dyserythropoiesis, and mature gametocyte prevalence, but not immature gametocyte prevalence (Aguilar et al., 2014). These findings indicate that in this patient cohort low hemoglobin levels do not enhance sexual commitment, but rather suggest that gametocyte maturation in the bone marrow may interfere with erythrocyte production resulting in lower hemoglobin levels. Further work is needed in larger, more diverse populations to evaluate this more carefully.

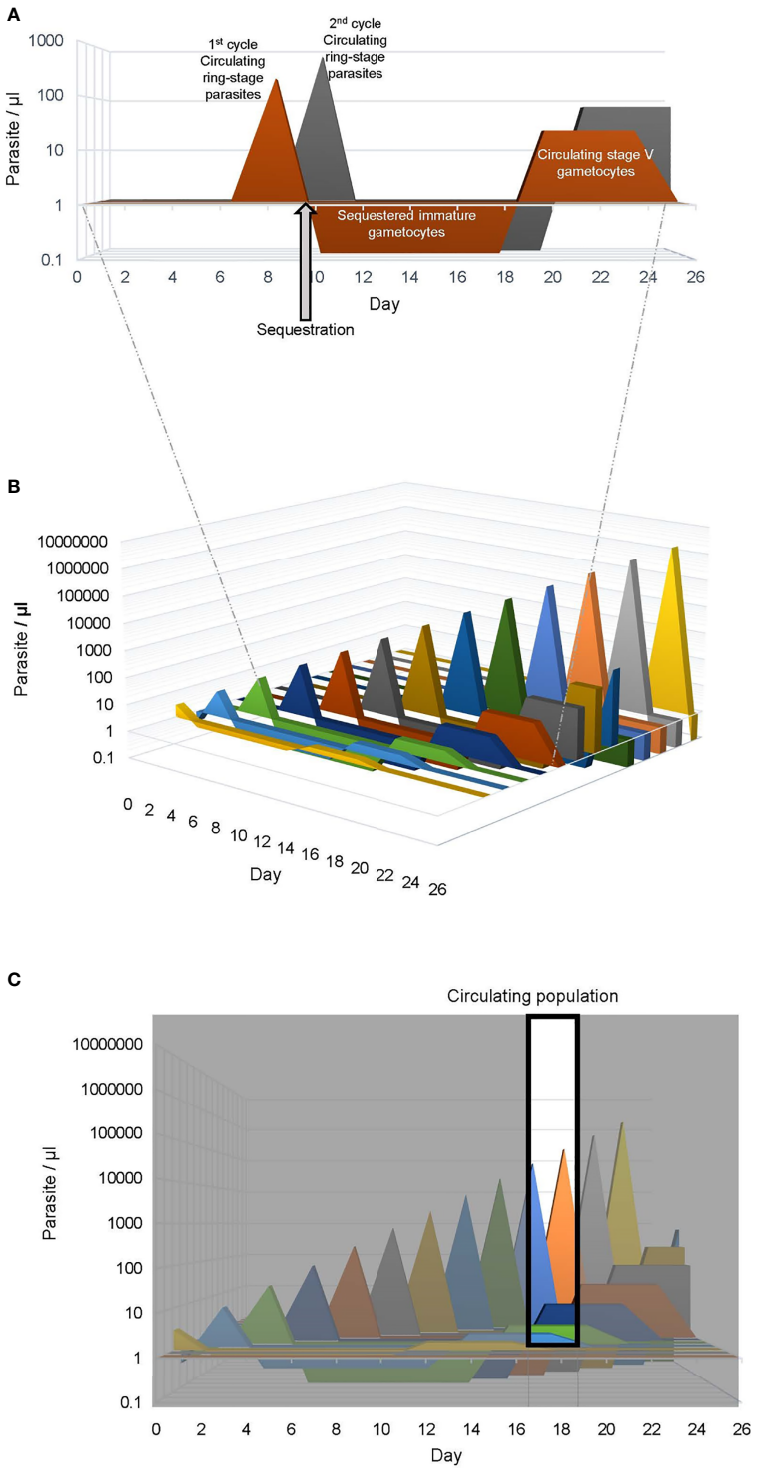

FIGURE 2 | Human *Plasmodium falciparum* infection dynamics. Graphical representation of *P. falciparum* infection dynamics in human peripheral blood assuming a multiplication rate of three and a 10% sexual commitment rate. **(A)** The asexual ring stage and sexual stage parasites produced from 2 consecutive life cycles (1st, orange and 2nd, gray) is shown. The parasitemia of the circulating ring-stage and mature stage-V gametocytes is indicated as a positive value on the vertical axis, while the sequestered immature gametocyte parasitemia is indicated as a negative value. For clarity the sequestered mature asexual parasitemia is not included. **(B)** The graph is expanded to depict the asexual and sexual parasites produced in 13 sequential life cycles, mimicking a 26 day *P. falciparum* blood stage infection. **(C)** The window indicates the population of parasites collected in a blood sample obtained between day 17 and 19, which would include circulating ring stage parasites < 30 hours post RBC invasion from the 10th and 11th life cycles and mature stage V gametocytes (>10 days post RBC invasion) from the first 4 life cycles.

To more directly evaluate clinical conditions closer to the time of initial sexual differentiation, blood samples from malaria patients were maintained in culture for 8 days in the presence of N-acetyl glucosamine (NAG) to block asexual growth and monitor gametocyte production (Usui et al., 2019). This approach revealed that 76% of the samples produced gametocytes, but the number of ring-stage parasites that differentiate sexually, referred to as the gametocyte conversion rate (GCR), varied from 0–78%. Gametocyte conversion correlated positively with the parasitemia of the initial ex vivo sample and a specific allele of a gene, *gdv1*, previously associated with gametocyte production *in vitro*. In contrast, fever and high lysophosphatidylcholine (LysoPC) levels had a negative influence. Neither patient hematocrit, age, nor leukocyte counts were associated with gametocyte commitment. The study also identified 3 molecular markers of sexual commitment in blood samples that can be used in place of the ex vivo assay facilitating future studies of larger cohorts to extend and refine the analysis of factors contributing to gametocyte commitment (Prajapati et al., 2020).

The *ex vivo* study described above was limited to subjects from a single site in Coastal Ghana and excluded HbS positive individuals. Therefore, neither human genetic variation nor environmental factors were evaluated although they have been associated with gametocyte production. It has been reported that people with HbAS, HbAC, and HbCC β-globin genotype have higher gametocyte densities and/or higher infection rates than those with the HbAA genotype (Robert et al., 1996; Gouagna et al., 2010). Lawaly et al. (Lawaly et al., 2010) also showed a significant human genetic contribution to gametocyte prevalence in individuals from family-based cohort studies from Senegal and Thailand. Intriguingly, this effect was apparent only in asymptomatic *P. falciparum* infections, not in symptomatic patients.

Climatic conditions that influence the growth and survival patterns of mosquito species with different vectorial capacities are additional factors that influence malaria transmission. In some areas, the prevalence of asexual parasites (Baird et al., 2002) and gametocytes (van der Kolk et al., 2003) have been reported to vary with season. Interestingly, in moderate and low transmission sites in western Kenya gametocyte levels are higher following the dry season than during the wet season when the prevalence of asexual parasites and clinical disease is high (Oduma et al., 2021). On the other hand, in coastal Ghana gametocyte prevalence increased during the dry season in an urban area, not a rural area (Ayanful-Torgby et al., 2018), demonstrating local variation and the need for large, long-term studies to understand the contributing factors. Higher expression of early gametocyte genes was also observed in regions with lower transmission levels when comparing sites in Kenya and Sudan (Rono et al., 2018) suggesting that parasites may adapt to the local transmission intensity. One hypothesis is that the parasite can sense the number of mosquito bites (Billingsley et al., 2005; Gadalla et al., 2016). However, there is no direct evidence for this association in humans or in rodent malaria models. In fact, in murine *P. chabaudi* and *P. vinckei* infections the gametocyte rate did not increase in response to mosquito probing (Shutler et al., 2005).

IN VIVO GAMETOCYTE LOCALIZATION AND DEVELOPMENT

Autopsy studies in the early 1900s identified immature gametocytes in the bone marrow and spleen, not other organs (Thomson, 1914), even though mature asexuals were found in other organs (Thomson, 1914; Garnham, 1931). The highest burdens of mature asexuals were seen in the brain and large intestines (Garnham, 1931), but this could vary depending on the pathology observed (Garnham, 1931). One hundred years later this finding was confirmed using molecular approaches to evaluate parasite burdens in autopsy samples from children that died of cerebral malaria. RT-qPCR analysis found the highest level of early gametocyte-specific transcripts in the bone marrow followed by the spleen (Joice et al., 2014). A higher level of immature gametocytes to mature gametocytes in the bone marrow versus peripheral blood has also been reported by Aguilar et al. (2014) and Smalley et al. (1981). Immunohistochemistry with a pan-gametocyte marker, anti-Pfs16 antibodies (Eksi et al., 2005), found the gametocyte/asexual parasite ratio significantly higher in bone marrow, even though total gametocyte levels were highest in brain and gut followed by the bone marrow, spleen, and heart (Joice et al., 2014). Subcellular localization studies in the bone marrow found equal numbers of gametocytes and asexuals in the parenchyma, while asexual parasites were more abundant intravascularly (Joice et al., 2014). A case study of a bone marrow sample from an adult malaria patient that had been treated with atovaquone, which cleared asexual parasites, also found immature gametocytes were more likely to be extravascular than mature gametocytes (Farfour et al., 2012). Similar localization studies have not been reported for the spleen which is a difficult biopsy but would be interesting.

The presence of extravascular parasites in the bone marrow raises several mechanistic questions. First, how did they get access? Second, does this environment enhance sexual differentiation? These questions will be addressed after a brief introduction to the bone marrow. The bone marrow is the primary site of hematopoiesis and produces 6 billion cells per kilogram of body weight per day (Davé and Koury, 2021) that enter the circulation by crossing the single layer of endothelial cells surrounding the vascular sinusoids. Egress occurs when blood cells mature to the point they no longer express the surface molecules needed to retain them in the bone marrow and the pressure gradient forces them through the endothelial layer into the vasculature (Waugh et al., 1984; Lichtman and Santillo, 1986; Beck et al., 2014). This egress model fits with studies demonstrating that immature gametocytes, stage II-IV are quite stiff and at physiologic pressures cannot pass through a matrix simulating the density of the spleen (Aingaran et al., 2012; Dearnley et al., 2012; Tibúrcio et al., 2012). Deformability increases as gametocytes transition to stage V gametocytes allowing them to move through the same matrix (Tibúrcio et al., 2012). Given the fenestrated endothelium of the spleen that allows passive trafficking of nonmotile RBC from the vasculature, it is possible that as gametocyte committed rings

mature and stiffen they become trapped in the spleen until they again become deformable at stage V. However, this does not explain entry to the extravascular space of the bone marrow.

Leukocytes and hematopoietic progenitor and stem cells expressing the appropriate adhesion molecules, CXCR4 and integrin α4β1 actively migrate into the bone marrow parenchyma in response to a CXCL12 gradient (Mazo et al., 2011), but the entry of RBCs has not been reported. One option for parasite access might be that a merozoite released by a schizont in the bone marrow sinusoids could bind to another cell as it enters the bone marrow, but this is speculation. Based on *in vitro* studies (Tamez et al., 2009; Neveu et al., 2020), once in the bone marrow parenchyma both asexually and sexually-committed merozoites could invade erythrocyte precursors developing there, starting from the late orthochromatic stage and complete development as the erythrocyte matures (**Figure 3**). If this is also the case *in vivo*, then after the infected cell matures it could be released into the circulation or the schizont could rupture in the bone marrow where the merozoites could reinvade another developing erythrocyte (**Figure 3**). It has been hypothesized that erythrcyte precursors preferentially select for sexual development (Peatey et al., 2013; Brancucci et al., 2017). However, there is strong ex vivo evidence that sexually-committed rings circulate for 13-20 hours before sequestration (Smalley et al., 1981; Usui et al., 2019; Prajapati et al., 2020). This has also been supported by transcriptomic data (Lemieux et al., 2009; Pelle et al., 2015; Prajapati et al., 2020).

Given the evidence for the circulation of sexually-committed rings, another alternative hypothesis for bone marrow access is the direct entry of RBCs infected with sexually or asexually-committed ring stage-parasites at least in the fatal malaria cases assessed by autopsy (Joice et al., 2014) and the anemic patients assessed by biopsy (Aguilar et al., 2014; Joice et al., 2014) (**Figure 3**). During malaria infection, there is an increase in granulocytes in the bone marrow, but a transient decrease in the numbers of lymphocytes, erythrocytes, and megakaryocytes (Villeval et al., 1990a; Villeval et al., 1990b). It is possible the inflammation induced by severe or chronic malaria infections

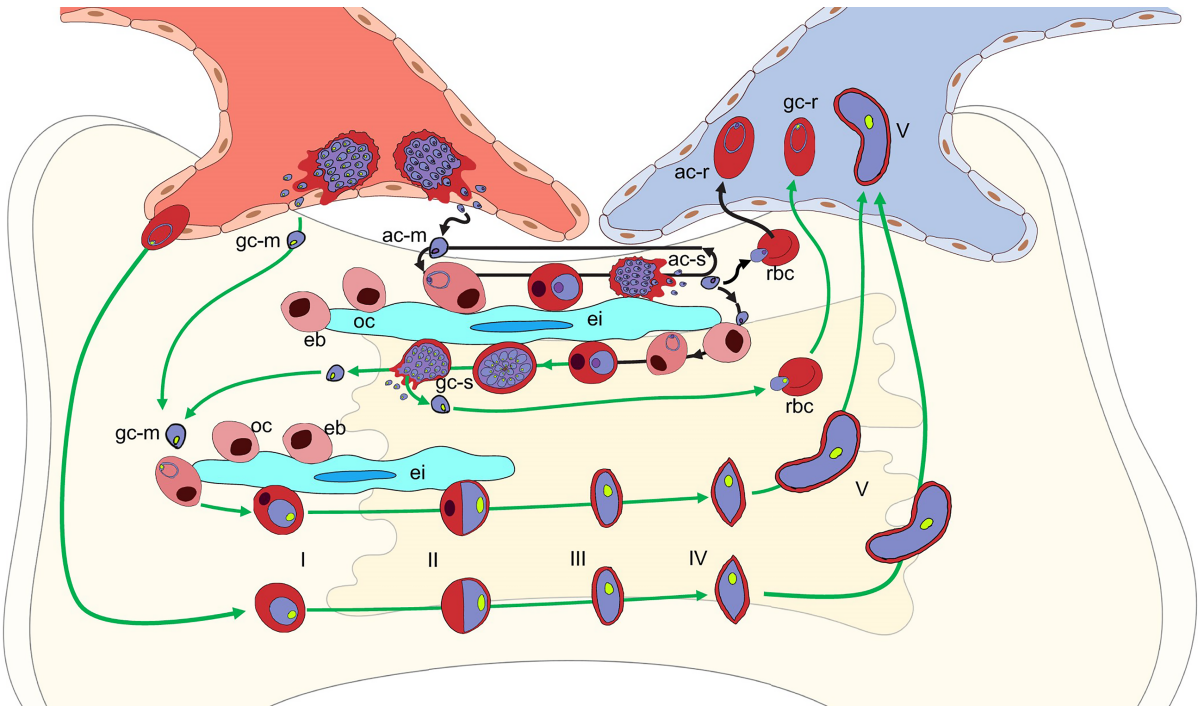

FIGURE 3 | Potential *P. falciparum* developmental pathways in the bone marrow parenchyma. Schematic of potential developmental pathways for asexually-committed (ac-m, purple nucleus), gametocyte committed (gc-m, green nucleus) merozoites, or erythrocytes containing ring stage parasites. The mechanisms involved in the parasite's exit from the circulation, across the endothelial cells into the bone marrow parenchyma remains unclear. Once in the parenchyma where erythroblastic islands (ei) support developing erythroblasts (eb), the ac-merozoite (black arrows) could invade a developing erythroblast after the orthochromatic stage (oc) and continue to develop into either an ac-schizont (ac-s) (main arrow) or, although not shown for clarity, a gc-schizont (gc-s). The ac-merozoite could also invade a newly formed RBC and be released back into the blood stream as a ring stage parasite (ac-r). Likewise, an ac-merozoite generated in the parenchyma could also have the same three fates and these are indicated in the figure. The gc-merozoite (green arrows) from the blood or produced in the bone marrow could invade a developing erythroblast after it transitions to the orthochromatic stage (oc) and continue to differentiate into a mature gametocyte (V) that is deformable enough to be released back into the blood stream. As with the as-merozoite, the gc-merozoite could also invade a newly formed RBC and be released back into the blood stream as a gc-ring stage parasite (gc-r). If erythrocytes containing gc-rings enter the bone marrow, they could also continue to develop through the stiff immature stages to a deformable mature stage V gametocytes that could be released to the circulation. Although not shown due to space constraints, if erythrocytes containing gc-rings enter it is likely that erythrocytes containing ac-rings could also enter and continue development into ac- or gc-schizonts.

may enhance the permeability of the endothelial cells of the sinusoids or the newly reported trans-cortical vessels (Aguilar et al., 2014; Grüneboom et al., 2019; Morikawa et al., 2020). Once in the parenchyma, the asexually or sexually-committed ring infected RBCs could be retained in the bone marrow during maturation by the expression of parasite encoded adhesion molecules on the surface of the infected RBC. Adhesion molecules, such as PfEMP1, localized to knob-like structures on the surface RBCs during asexual parasite maturation are known to be involved in sequestration to endothelial cells as well as syncytiotrophoblasts in the placenta (Leech et al., 1984; Rogers et al., 1996; Rogers et al., 2000). Sexually-committed parasites do not produce knob-like structures and have not been shown to adhere to endothelial cells but do produce a set of gametocyte-specific proteins that are predicted to be exported to the RBC (Eksi et al., 2005; Silvestrini et al., 2010; Tibúrcio et al., 2013). These proteins could be involved in adhesion at least until the gametocyte stiffens enough to prevent egress before maturation to stage V. There is also evidence for both asexually- and sexually-committed parasites binding to mesenchymal cells, but the specific parasite-produced proteins have not been identified (Messina et al., 2018). Inside the bone marrow, the parasites could continue sexual or asexual development and be released into the circulation as a stage V gametocyte, merozoite, or newly invaded developing RBC (**Figure 3**). The merozoites could also reinvade a developing erythrocyte in the bone marrow and begin asexual or sexual development (**Figure 3**).

Although as discussed above, there is no direct support that bone marrow or spleen environments enhance commitment to sexual differentiation, it is possible that their unique environments are optimal for gametocyte maturation. The relative distribution of fatty acids, including LysoPC, differ considerably from that in the serum (Brancucci et al., 2017). Studies in mice combining *in vivo* imaging with direct measurement of local concentration of oxygen (pO_2) in the calvarial bone marrow suggest that the pO_2 inside blood vessels sharply drops after vessels enter the bone marrow, an observation attributable to active O_2 consumption by bone marrow cells (Spencer et al., 2014) and the association of hypoxia with hematopoietic stem cell quiescence (Hermitte et al., 2006; Eliasson and Jönsson, 2010). It is difficult to test this directly in humans, but the recently validated biomarkers for early sexually-committed ring-stage parasites can be used to assess the *in vivo* relationship between sexually-committed-rings and circulating gametocyte levels 10-11 days later. This information can be used to determine the association between gametocyte maturation *in vivo* and different clinical factors, including hematopoiesis, parasitemia, and fever as well as other factors recently found to influence gametocytogenesis *in vitro*.

MOLECULAR MECHANISMS CONTRIBUTING TO GAMETOCYTOGENESIS *IN VITRO*

At the molecular level, *P. falciparum gametocyte* development 1 (*gdv1*, PF3D7_0935400) was the first gene found to play a major role in gametocyte production followed by the identification of ApiAP2 family transcription factor (*ap2-g*, PF3D7_1222600), which is regulated by GDV1 (Eksi et al., 2012; Filarsky et al., 2018; Usui et al., 2019). The loss of gametocyte production in lab strains is frequently associated with *Gdv1* deletions (Tiburcio et al., 2021), while GDV1 overexpression increases gametocyte production (Eksi et al., 2012; Filarsky et al., 2018). Field populations also exhibit high *gdv1* allelic divergence that has been suggested to vary with transmission patterns (Mobegi et al., 2014; Bungei et al., 2020). *Ap2-g* was also identified as the target of mutations in gametocyte-deficient parasite lines in both *P. falciparum* (Kafsack et al., 2014) and *P. berghei* (Sinha et al., 2014). During the asexual replication cycle *ap2-g* expression is silenced. Once *ap2-g* transcription is induced and AP2-G protein levels increase it enhances its own transcription (Kafsack et al., 2014; Sinha et al., 2014; Poran et al., 2017) and initiates gametocytogenesis through a cascade of gene expression that is tightly controlled through complex regulatory systems (Poran et al., 2017; Josling et al., 2018). A*p2-g* is epigenetically silenced by histone 3 lysine 9 trimethylation (H3K9Me3), which is stabilized by heterochromatin protein 1 (*hp1*, PF3D7_ 1220900) binding. Histone deacetylase 2 (*hda2*, PF3D7_1008000) which removes the acetyl group from H3K9 facilitating methylation has also been shown to affect gametocyte production (Brancucci et al., 2014; Coleman et al., 2014). This work has been described in detail in a number of recent reviews (Josling et al., 2018).

Although identified in *in vitro* culture adapted parasite lines, strains lacking *gdv1* and *ap2-g* have not been identified in the field. This difference is likely due to the need for gametocytes for transmission from person to person *via* a mosquito in the field, which is not required for propagation in culture. In fact, propagation in culture selects for gametocyte-deficient strains that produce more asexually-committed merozoites, instead of both asexually and sexually-committed merozoites. Despite the strong evidence that *gdv1* and *ap2-g* play major roles in gametocytogenesis, *gdv1+/ap2g+ P. falciparum* strains can have markedly different sexual differentiation rates (0 to 20%) (Brancucci et al., 2015; Poran et al., 2017) suggesting additional genetic factors play a role. There is also marked intra-strain variation in gametocytogenesis implying environmental modulation.

IN VITRO GAMETOCYTE PRODUCTION

In *P. falciparum*, several extracellular factors such as nutritional level, parasitemia, oxidative stress, cAMP, cGMP or lactate levels, and drug treatment have been reported to influence gametocyte conversion in *in vitro* culture (Buckling et al., 1999; Price et al., 1999; Sowunmi et al., 2006; Chaubey et al., 2014; West and Sullivan, 2020). However, using traditional *in vitro* culture methods the low yield of gametocytes and intra-strain variation, which can range from 0-3% gametocytemia, have made the reports difficult to replicate (See Buchholz et al., 2011, **Supplemental Data 1C**). Consequently, the specific stressors and signaling pathways that connect these environmental signals to parasite development have been elusive and instead lead to the general notion that stress

induces gametocyte production (Dyer and Day, 2000; Baker, 2010; Josling et al., 2018).

Recent work by Brancucci et al. has advanced our understanding of the extracellular factors involved in regulating asexual growth and sexual differentiation. They identified a parasite line tagged with an early gametocyte reporter, Pf2004/164tdTom, that required serum-free media to make gametocytes. Using this line, they carefully fractionated serum components and found that the addition of LysoPC effectively reduced gametocyte production and increased asexual replication to the levels observed in media with serum (Brancucci et al., 2017). Tracking the metabolism of labeled LysoPC demonstrated that extracellular LysoPC is the parasite's primary source for choline for the production of phosphatidylcholine (PC) (Pessi et al., 2004). PC is a major component of phospholipid membranes in all eukaryotic cells and is required for the production of the 16- 32 new merozoites every 48 hours. The mechanisms used to transport LysoPC from the plasma into the intraerythrocytic parasites remain unknown, but once inside the parasite the results demonstrate that the choline moiety is removed from LysoPC and used to synthesize PC *via* the Kennedy Pathway (Gibellini and Smith, 2010). When LysoPC is depleted from the media the parasite can survive for one asexual cycle by using phosphoethanolamine methyltransferase (PMT) to produce phosphocholine (P-choline) by trimethylation of parasite produced phosphoethanolamine (P-ethanolamine (Witola et al., 2008; Serrán-Aguilera et al., 2016; Brancucci et al., 2018). However, the number of new merozoites produced is reduced which results in fewer new ring-stage parasites (Brancucci et al., 2017). Interestingly, in the absence of LysoPC a higher proportion of the ring stage parasites produced are committed to sexual differentiation. Gametocyte commitment varies for different parasite strains, but even in the absence of LysoPC remains below 30% of the ring stage parasites. This indicates that some ring-stage parasites continue to replicate asexually in the absence of LysoPC, but the mechanism regulating this remains unknown. This differential response to the same media is consistent with environmental factors modulating, not triggering, sexual differentiation.

One proposed mechanism for the regulation of sexual conversion in the absence of LysoPC is lowering the intracellular levels of S-adenosylmethionine (SAM). SAM is the substrate PMT uses to trimethylate P-ethanolamine to produce P-choline for the synthesis of PC. SAM is also used to methylate histones. Since sexual conversion is repressed by histone trimethylation mediated silencing of *ap2-g*, it has been suggested that the increased use of SAM to trimethylate P-ethanolamine could reduce histone methylation thereby enhancing *ap2-g* transcription and sexual conversion (Llinás, 2017). This is an intriguing hypothesis and awaits further experimental testing. The feedback pathway that regulates the upregulation of PfPMT in response to low choline levels is also unknown.

DISCUSSION

The dual role of LysoPC depletion in reducing asexual growth and enhancing gametocyte production complicates dissecting the specific mechanisms involved. It also highlights the need for careful assessment of both asexual growth and gametocyte production. Both are interrelated because during each asexual cycle both asexually and sexually committed-schizonts, -merozoites, and -newly invaded rings are formed. If a treatment affects asexual growth so that the treated culture forms fewer new parasites, the number of gametocytes produced would also be lower. It is relatively easy to control for this by using gametocyte conversion rate (GCR) instead of just gametocytemia to monitor gametocyte conversion (**Table 1**). The GCR is calculated by dividing the stage II gametocytemia on day (D) 4 or stage III gametocytemia on D6 by the total ring/trophozoite stage parasites on D0-D1 before blocking asexual replication using NAG (**Table 1**, column H). Asexual replication should be blocked using NAG because subsequent asexual cycles also produce sexually-committed gametocytes, which can complicate quantification. This continuous gametocyte production also means that gametocytes generated during earlier cycles are also present and need to be excluded during quantification. This is a particular problem if the culture has been maintained at a high parasitemia for several cycles and can result

TABLE 1 | Gametocyte conversion calculation table.

	B	C	D	E	F	G	H	I	J
1	Group	Set up	Initial parasitemia cycle			G-cyte	G-cyte conversion		
2		Parasitemia (%)	Next cycle (D0) parasitemia (%)	Multiplication rate (MR)	Control MR	D4 or D6 g'cytemia (%)	Original GCR (%)	RBC-rupture-corrected GCR (%)	Control MR-corrected GCR[#] (%)
3*				=D/C	=E4		=(G/D) *100	=((G/(100-D)*100)/D) *100	=((G/(100-D))*100)/(C*F)*100
4	Control	2.00	8.68	4.34	4.34	0.87	10.02	10.98	10.98
5	Pseudo-induction	2.00	5.19	2.59	4.34	0.90	17.34	18.29	10.94
6	Induction	2.00	8.19	4.09	4.34	1.88	22.95	25.00	23.59

**Row 3: The formulas provided can be used to calculate the different gametocyte conversion rates (GCR) for different experimental conditions from the set up (column C) and D0 asexual cycle parasitemias (column D), the control multiplication rate (column F), and the gametocytemia (column G).*

[#]Row 5: If the control-corrected GCR (column J) is the same as the RBC rupture-corrected GCR for the Control group (column I & J, row 5) then additional experiments are needed to confirm that the production of asexually-committed, D0 rings was not preferentially affected by the induction conditions.

in a high gametocyte background. One way to control for this is to only count stage II gametocytes on D4 or stage III on D6.

One major caveat to the use of the GCR for *in vitro* cultures is the observation that asexually replicating parasites die or "crash" (Saliba and Jacobs-Lorena, 2012; Delves et al., 2016) when parasitemia rises much above 5-6% while developing gametocytes are more resistant and can continue to mature. The differential resistance of asexually replicating and sexually differentiating parasites to crashing was the original strategy used to isolate gametocytes. The asexual parasitemia was allowed to increase in the culture until they all died, leaving a mixed population of gametocytes at different developmental stages. The specific factors that contribute to parasite death have not been identified, but Chou et al., (2018) showed that death began before schizogony. However, whether this differs for asexually and sexually-committed schizonts has not been tested. If asexually-committed schizonts are preferentially affected then the total number of rings would decrease, while the number of sexually-committed rings remains the same, thereby increasing the GCR in the absence of an actual increase in sexual conversion.

Asexual and gametocyte stages are also known to differ in their sensitivity to drugs that target DNA replication (Buchholz et al., 2011; Collins et al., 2019) and sorbitol lysis (Saul et al., 1990). Sorbitol resistance suggests a difference in permeability, perhaps due to the lack of the parasite-specific anion channel (PSAC), but this has not been definitely demonstrated (Saul et al., 1990; Nguitragool et al., 2011). This lower permeability could reduce the uptake and response of gametocytes to compounds in the media and again lead to a preferential decrease in asexually-committed, not sexually-committed parasites leading to an apparent increase in GCR. One option to control for this is to monitor the number of merozoites produced and the asexual multiplication rate. The asexual multiplication rate is the increase in parasitemia from one asexual generation to the next. Both the total number of merozoites produced and their invasion rate contribute to the multiplication rate. If conditions alter the multiplication rate, as observed when the culture crashes, then further analysis is needed to differentiate between alterations in asexual and/or sexual differentiation (**Table 1**). Another alternative is to quantify the total number of schizonts, newly invaded rings, and gametocytes produced instead of using parasitemia and gametocytemia. However, this remains technically challenging due to errors introduced during the large dilution needed to count individual RBCs. The lack of markers for sexually-committed schizonts and merozoites also complicates the analysis.

To facilitate tracking the transition from schizonts to gametocytes and determine whether treatment alters parasite growth and/or sexual commitment, a spreadsheet was developed (**Table 1**). The spreadsheet uses initial parasitemia for each flask, the ring/trophozoite parasitemia of the next asexual cycle (D0) to calculate the multiplication rate and the D0 parasitemia and stage II gametocytemia on D4 or stage III gametocytemia on D6 to calculate the GCR. The observed gametocytemia is then corrected for the loss of infected RBC during schizont rupture (RBC-corrected GCR, **Table 1**, column I), which can be substantial at high starting parasitemias. If the test culture has a different multiplication rate than the control culture, then the GCR for the test culture can also be recalculated using the number of rings that would have been produced with the control multiplication rate (MR). If this control MR-GCR (**Table 1**, column J) now equals the GCR of the control group, further analysis is required to confirm whether the original GCR was due to an increase in sexual conversion or a selective decrease in asexually-committed parasites. This detailed analysis of both asexual and gametocyte production should facilitate the identification of the sources of variability in gametocyte production, allowing more accurate and reproducible detection of induction conditions and ultimately replacing "stress" with actual regulatory mechanisms.

SUMMARY

Key genes, *ap2-g*, *gdv1* and *hp1*, involved in the initiation of *P. falciparum* sexual differentiation have been identified, but the mechanisms regulating induction in just a subpopulation of developing schizonts remains a mystery. Inter and intra-strain differences in sexual commitment in wild type (*ap2-g+/gdv1+/hp1+*) parasites suggest additional genetic and environmental factors play a role. Sensitive biomarkers for sexually-committed ring-stage parasites coupled with careful assessment of parasite growth and differentiation *in vitro* provide tools to extend the analysis to a range of environmental factors. Of particular interest are the roles of LysoPC and bone marrow and spleen sequestration, as well as quantifying gametocyte-committed rings and their maturation to gametocytes in asymptomatic and symptomatic individuals. Understanding the factors contributing to the infectious reservoir will be important to design control strategies for malaria elimination.

AUTHOR CONTRIBUTIONS

MU and KW both conceived of, researched, and wrote the manuscript. All authors contributed to the article and approved the submitted version.

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Plasmodium falciparum and *Plasmodium vivax* Adjust Investment in Transmission in Response to Change in Transmission Intensity: A Review of the Current State of Research

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Malaria parasites can adjust the proportion of parasites that develop into gametocytes, and thus the probability for human-to-vector transmission, through changes in the gametocyte conversion rate. Understanding the factors that impact the commitment of malaria parasites to transmission is required to design better control interventions. *Plasmodium* spp. persist across countries with vast differences in transmission intensities, and in sites where transmission is highly seasonal. Mounting evidence shows that *Plasmodium* spp. adjusts the investment in transmission according to seasonality of vector abundance, and transmission intensity. Various techniques to determine the investment in transmission are available, i.e., short-term culture, where the conversion rate can be measured most directly, genome and transcriptome studies, quantification of mature gametocytes, and mosquito feeding assays. In sites with seasonal transmission, the proportion of gametocytes, their densities and infectivity are higher during the wet season, when vectors are plentiful. When countries with pronounced differences in transmission intensity were compared, the investment in transmission was higher when transmission was low, thus maximizing the parasite's chances to be transmitted to mosquitoes. Increased transmissibility of residual infections after a successful reduction of malaria transmission levels need to be considered when designing intervention measures.

Keywords: *Plasmodium falciparum* (Pf), *Plasmodium vivax* (pv), gametocyte carriage, investment in transmission, seasonality, transmission intensity

INTRODUCTION

Transmission of malaria parasites from the human to the mosquito host is a crucial bottleneck in the lifecycle of the parasite. Not all *Plasmodium* species infections contribute to transmission. Infection of the mosquito depends on the presence of the sexual forms of the parasite, male and female gametocytes, in the blood, and their uptake by mosquitoes.

Over the course of the *Plasmodium* spp. intraerythrocytic 48-h cycle, only a small proportion of parasites commit to sexual differentiation and develop into gametocytes (Sinden, 1983). Once taken up by mosquitoes, male and female gametocytes fuse to form oocysts. Approximately 14 days after the uptake of gametocytes, infective sporozoites are present in the mosquito's salivary glands (Meis et al., 1992), resulting in human infection once the next blood meal is taken.

Plasmodium falciparum (*P. falciparum*) and *Plasmodium vivax* (*P. vivax*) are the primary cause of malaria in humans. Both species combined present the greatest threat, accounting for the majority of malaria related morbidity and mortality. *P. falciparum* is responsible for most cases of severe clinical malaria. The duration of gametocyte development and maturation differ between *P. falciparum* and *P. vivax*. *P. falciparum* gametocyte development takes 8-12 days, during which gametocytes undergo five morphologically distinct stages (Hawking et al., 1971; Sinden et al., 1978). Immature stages (late stage I-IV) sequester in inner organs and are absent from blood circulation i.e., they sequester in inner organs until maturity (Paul et al., 2000; Eichner et al., 2001; Farfour et al., 2012). As a result of the long maturation period, gametocytes are often not present during the first wave of asexual parasitemia when clinical malaria occurs. In contrast, *P. vivax* gametocytes take only 2-3 days for maturation, and infective gametocytes are present in circulation before clinical symptoms occur (Boyd and Stratman-thomas, 1934; Boyd et al., 1936; Vallejo et al., 2016).

The switch between asexual development and sexual differentiation is one of the few times in the parasite's lifecycle that it can adjust its strategy to maximize fitness. The parasite can prioritize either to produce more gametocytes (i.e., increase the conversion rate) to increase its chances of transmission, or increase replication of asexual parasites to enhance its survival in the host (Reece et al., 2009; Carter et al., 2013). Gametocytes that are not taken up by mosquitoes cannot return to asexual development, thus the investment in transmission will be lost.

The proportion of parasites that develop into gametocytes, i.e., the gametocyte conversion rate, differs substantially between isolates collected from patients in the field (Smalley et al., 1981; Usui et al., 2019). It might vary over the duration of untreated infections, and be adjusted in response to antimalarial treatment or other external factors (Schneider et al., 2018). Also, the duration of gametocyte circulation in peripheral blood might vary (Eichner et al., 2001), further adding to variation of transmission potential. Understanding factors (i.e., the stresses) that impact the transmission potential is critical to guide and evaluate transmission reducing interventions.

As many countries transition from malaria control to elimination, the focus shifts from diagnosis and treatment of clinical cases to understanding the full transmission reservoir, including transmission originating from subclinical infections (Bousema et al., 2014). Various interventions, e.g. vector control methods such as use of bed nets, or indoor residual spraying, and parasite screening and treatment have been key to reducing transmission. An increasing number of studies indicate that parasites adjust their investment in transmission in response to a changing environment (Mobegi et al., 2014; Gadalla et al., 2016; Ouédraogo et al., 2016; Parobek et al., 2016; Rono et al., 2018; Koepfli et al., 2021; Oduma et al., 2021). A better understanding of these processes is needed to develop novel strategies to achieve elimination. Identifying factors that result in higher investment to transmission might allow control programs to tailor their interventions towards covering these factors.

Here, we summarize the methodology to measure the commitment to transmission in natural infections, and review recent field studies assessing the impact of changes in transmission intensity across seasons or long-term (i.e., between countries differing in transmission) on the commitment to transmission and infectivity of *P. falciparum* and *P. vivax*.

DIRECT AND INDIRECT MEASURES OF COMMITMENT TO TRANSMISSION

In recent years, much has been revealed about the molecular basis of gametocyte conversion. The underlying processes have been reviewed elsewhere (Josling and Llinás, 2015; Nilsson et al., 2015; Josling et al., 2018). In brief, the Apetala-2 transcription factor (Ap2-g) is essential for the differentiation of gametocyte-committed parasites into early gametocytes (Kafsack et al., 2014). Expression of *ap2-g* is activated by interactions of nuclear proteins *P. falciparum* gametocyte development protein 1 (GDV1) and heterochromatin protein 1 (HP1) (Eksi et al., 2012; Brancucci et al., 2014; Filarsky et al., 2018). In asexual parasites, the *Ap2-g* is epigenetically silenced (Flueck et al., 2009; Lopez-Rubio et al., 2009; Brancucci et al., 2014; Coleman et al., 2014).

In field isolates, commitment to transmission can be assessed through various methods. The most direct measure of the conversion rate can be obtained in short-term culture, i.e., by quantifying early gametocytes after 2-8 days in culture by microscopy and comparing their density to parasite density at day 0. These assays require laboratories for parasite culture to be present in the study site and are complex to perform, thus comparably few studies have adopted this method.

Studies assessing *P. falciparum* gametocyte conversion directly have found pronounced differences among isolates (Smalley et al., 1981; Poran et al., 2017; Usui et al., 2019; Prajapati et al., 2020). An early study in 1981 in Ghana found a mean gametocyte conversion rate of 7.6% among infections that already carried mature gametocytes, and of 1.3% among infections without any mature gametocytes detected by microscopy (Smalley et al., 1981). A more recent study applied similar methodology to measure the gametocyte conversion rate in 260 children presenting with clinical malaria in Ghana. The gametocyte conversion rate was determined microscopically by counting the number of early stage gametocytes at day 4 or 8 after culture and compare it to asexual parasite density at day 0. Three quarters of all isolates had detectable circulating gametocyte-committed rings. The gametocyte conversion rate varied widely, with up to 78% of all rings being committed to

sexual development. The median conversion rate was low at 0.7%, and 20% of the samples had high conversion rates of >4% (Usui et al., 2019). Due to the challenges in culturing *P. vivax*, no studies have attempted to measure *P. vivax* conversion using similar methodology.

As an alternative to the direct measurement of the gametocyte conversion rate, many studies have quantified mature gametocytes in human blood samples by microscopy, or through measuring expression of gametocyte-specific mRNA transcripts by RT-qPCR, NASBA, or other molecular methods. (Koepfli and Yan, 2018; Tadesse et al., 2019). Molecular methods allow for quantification of very low-density parasites and gametocytes. Multiple molecular markers for gametocyte detection have been identified. Frequently used makers include *pfs25*, *pfs16*, *pfg377*, and *pfs230p* for *P. falciparum*, and *pvs25* (or *P. vivax*) [reviewed in (Koepfli and Yan, 2018)] Most of these genes are transcribed in the gametocyte, but translationally repressed until uptake of gametocytes by the mosquito (Mair et al., 2006; Guerreiro et al., 2014).The *Pfs25 and Pvs25* are the most widely used targets for gametocyte detection and quantification as they show limited polymorphism, are sensitive and are highly abundant in blood (Kaslow et al., 1989; Beurskens et al., 2009; Feng et al., 2011; Schneider et al., 2015). As in studies that measured conversion directly, large variation in the proportion of gametocytes compared to asexual parasites was found (Koepfli et al., 2015; Almeida et al., 2018; Tadesse et al., 2018; Kosasih et al., 2021).

While gametocyte quantification is a useful surrogate marker of infectivity, it is only an indirect measure of commitment to transmission (Koepfli and Yan, 2018). A direct comparison revealed a poor correlation between the gametocyte conversion rate and the proportion of mature gametocytes (Usui et al., 2019). Parasite densities in natural infections fluctuate across many orders of magnitude, thus differences in gametocyte densities might simply represent variation in parasite densities. Further, developing *P. falciparum* gametocytes sequester for approximately 10 days. Thus, gametocyte densities at the day of sampling might represent parasite densities up to two weeks previously. These densities are not known unless frequent follow-up sampling is conducted. *P. vivax* gametocytes develop within 2-3 days, thus gametocyte density might mirror commitment to transmission more directly.

Mosquito feeding assays are a further means to measure transmission potential. They are the gold standard to measure infectivity. Yet, as gametocyte densities, these studies measure commitment to transmission only indirectly. Infectivity can be measured as the percentage of human hosts that infect at least one mosquito, the proportion of mosquitoes infected, or the number of oocysts per infected mosquito. While gametocyte densities can serve as useful predictor for transmission potential, among individuals with gametocytes infectivity can be reduced in the presence of transmission-blocking immunity (Bousema et al., 2011), in case of an incompatibility between the parasite strain and vector species (Molina-Cruz et al., 2015; Tang et al., 2020), or if gametocytes are present, but not infective, e.g. after drug treatment (Abay, 2013), or when gametocytes are present but are not fully infective because mature gametocytes in peripheral circulation require additional 3 days to become fully infective (Smalley and Sinden, 1977; Lensen et al., 1999). While studies conducting feeding assays have overall shown a relationship between gametocyte density and the number of mosquitoes infected, infectivity varied among isolates. Even at high gametocyte densities, some blood samples did not result in all mosquitoes being infected, while some samples with very low gametocyte density were able to infect mosquitoes (Ouédraogo et al., 2016; Gonçalves et al., 2017; Bradley et al., 2018). Failure to infect mosquitos despite high gametocyte densities might be the result of transmission blocking immunity (i.e. antibodies in the human blood rendering gametocytes non-infective (Stone et al., 2018), or defense mechanisms of the mosquito (Cirimotich et al., 2010).

IMPACT OF TRANSMISSION INTENSITY ON TRANSMISSION POTENTIAL

Mounting evidence suggests that malaria parasites adjust their investment in transmission in response to transmission intensity in their environment, e.g. vector abundance (Gadalla et al., 2016; Ouédraogo et al., 2016; Parobek et al., 2016; Rono et al., 2018; Vantaux et al., 2018; Koepfli et al., 2021; Oduma et al., 2021). Such adaptations are both long-term, i.e., between countries differing in transmission, and short term, i.e., across seasons.

Adjustments to differences in transmission intensity in space and time are reflected in the genome and transcriptome of malaria parasites. A study compared whole genome sequencing data from parasites from the Gambia, where transmission is low and seasonal, and Guinea, where transmission is high (Mobegi et al., 2014). *Gdv1*, which is key for early gametocyte development, stood out as one of the genes that differed most between populations (Mobegi et al., 2014). This likely represents selection for alleles adjusted to the respective transmission intensity. In Cambodia, intensified control resulted in an 80% reduction in the number of *P. falciparum* cases from 2009 to 2013. The number of *P. vivax* cases increased in the same period (Maude et al., 2014). Genomic data provided a possible clue to the reasons for this increase. *P. vivax* parasites collected over this period were sequenced, and the strongest selective sweep was found around the *ap2-g* transcription factor (Parobek et al., 2016). This suggests that *P. vivax* adjusted its investment in transmission in response to control within only a few years, resulting in higher levels of transmission despite intensified vector control.

Similar results of adaptation in response to transmission intensity were observed in gene expression studies. A study compared *P. falciparum* isolates from non-immune children with clinical malaria from three sites in East African with long-term differences in transmission intensities, i.e., Kisumu, Kenya (high), Kilifi, Kenya (medium), and Sudan (low). Expression levels of *P. falciparum ap2-g* differed substantially between parasites isolated from the high *versus* low transmission settings. The expression levels of *ap2-g* increased as transmission intensity

decreased (Rono et al., 2018). These findings imply that in areas where malaria transmission is low, *P. falciparum* parasites invest more in transmission compared to areas where malaria transmission is high. This plasticity allows parasites in natural populations to adapt to their local environment to maintain fitness.

In another study comparing transmission potential among sites, *P. falciparum* and *P. vivax* gametocyte densities were compared in over 16,000 asymptomatic individuals in Papua New Guinea (PNG), Solomon Islands, Thailand, and Brazil (Koepfli et al., 2021). Thailand and Brazil had seen extended periods of very low transmission, potentially allowing parasite populations to adjust transmission strategies. The surveys in Papua New Guinea and Solomon Islands were conducted at times where transmission was moderate-high, or had recently been reduced. The proportion of infections with gametocytes detectable by RT-qPCR varied greatly among surveys, from 43% to 94% for *P. falciparum*, and from 23% to 78% for *P. vivax*. The proportion of gametocyte-positive infections was highest in regions with lowest transmission intensity, i.e., in Brazil, Thailand, and Solomon Islands for *P. falciparum*, and in Brazil and Thailand for *P. vivax*. In parallel, gametocyte densities and the proportion of gametocytes among all parasites tended to be higher where transmission had been low for extended periods.

The combination of differences in mean *P. falciparum* and *P. vivax* parasite densities and in the investment in transmission resulted in pronounced differences in the proportion of gametocyte carriers that could be detected by microscopy (**Figure 1**). Across surveys, 37% to 100% with *P. falciparum* gametocytes detected by RT-qPCR, and 42% to 84% of *P. vivax* gametocyte carriers were positive by microscopy (**Figure 1**) (Koepfli et al., 2021). Where *P. falciparum* prevalence was very low, i.e., in Brazil and Solomon Islands, most gametocyte carriers were positive by microscopy. These infections could thus be diagnosed by mass screen and treat programs (Koepfli et al., 2021).

The comparison of results from mosquito feeding assays across several sites also pointed adaptations to transmission intensity. Across three sites differing in *P. falciparum* transmission intensity, 1209 feeding experiments were conducted. A total of 39 individuals infected at least one mosquito. In Burkina Faso, where transmission was highest, almost all (25/27) individuals that could infect mosquitoes were positive for asexual parasites or gametocytes by research-grade microscopy. In Kilifi and Mbita, Kenya, where transmission is lower, 1/3 and 2/9 P. *falciparum* infectious individuals were submicroscopic. While these numbers are low and the differences do not reach statistical significance, the higher infectivity of submicroscopic infections in low-transmission settings might point to a higher proportion of gametocytes among all parasites, and thus might indicate a higher gametocyte conversion rate (Gonçalves et al., 2017).

IMPACT OF SEASONALITY ON TRANSMISSION POTENTIAL

In many malaria-endemic countries transmission occurs primarily during the wet season, when vectors are plentiful. Increasing the investment in gametocytes in the transmission season offers optimal fitness to the parasite population as it maximizes chances of onward transmission. Indeed, changes in the commitment to transmission were found across seasons in countries where malaria transmission is seasonal.

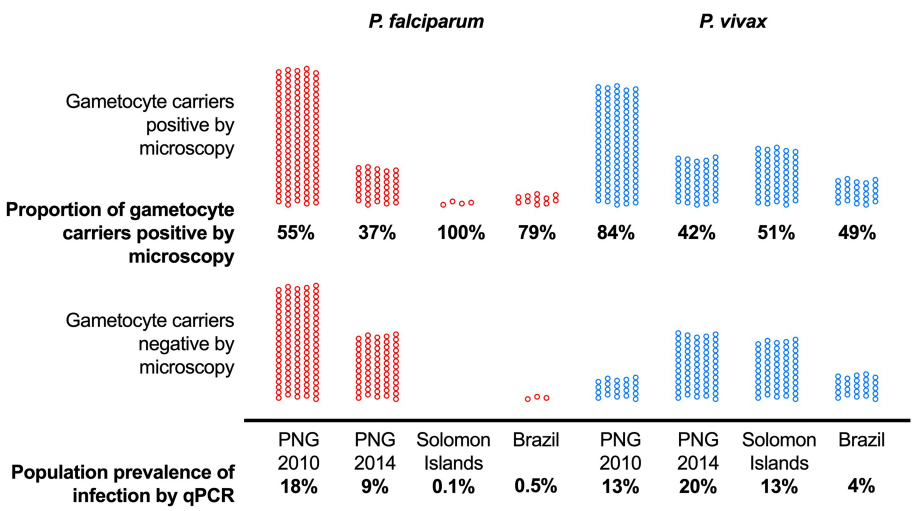

FIGURE 1 | The proportion of asymptomatic *P. falciparum* and *P. vivax* infections with gametocytes (detected by RT-qPCR) that can be detected by microscopy differs among sites with different transmission intensity. Data from 10,111 surveyed individuals included. Population prevalence of infection by qPCR is indicated at the bottom and ranged from 0.1% to 18% for *P. falciparum* and 4% to 20% for *P. vivax*. Among all *P. falciparum* gametocyte carriers, 37% to 100% could be identified by microscopy, and 42% to 84% of *P. vivax* gametocyte carriers. PNG, Papua New Guinea. Data from (Waltmann et al., 2015; Koepfli et al., 2017; Almeida et al., 2018; Koepfli et al., 2021).

Two studies have compared *P. falciparum* gametocyte densities and parasite densities in the wet and dry seasons. In western Kenya, a total of nearly 3000 individuals were sampled in the wet and the dry seasons to screen for *P. falciparum*. Infections were diagnosed by qPCR, and gametocytes quantified by RT-qPCR. Prevalence of infection differed only moderately between seasons, it increased from 13.5% in the dry season to 17.5% in the wet season. Mean parasite density was below 10 parasites/μL, and did not differ between seasons. A lower proportion of infections carried gametocytes in the wet season, but *pfs25* transcript densities were over 3-fold higher (**Figure 2**) (Oduma et al., 2021). In the dry season, very few individuals carried gametocytes that likely could infect mosquitoes. In the wet season, this number was much higher. The increase in gametocyte density while asexual parasite density changed little reflects an increase in the proportion of gametocytes among all parasites, and thus a possible indication of an increased investment in transmission.

A similar result was found in Sudan, where *P. falciparum* gametocytes densities were measured by RT-qPCR in 25 individuals that sustained chronic, asymptomatic infections across two transmission seasons. Gametocyte densities were higher in the period just before the main transmission season (i.e., when *Anopheles* mosquitoes are present but no clinical cases are reported), compared to the preceding transmission-free season (i.e., when neither vectors are present nor clinical cases are reported). As in the study in Kenya, *P. falciparum* parasite density did not change between the two seasons, suggesting an increase in the gametocyte conversion rate (Gadalla et al., 2016).

Several studies compared mosquito infectivity from *P. falciparum* asymptomatic carriers between seasons. In Burkina Faso, 130 individuals were randomly selected and gametocytes and infectivity quantified in the dry season, the beginning of the wet season, and the peak of the wet season. *P. falciparum* Parasite prevalence (84%-94% by molecular diagnosis) and gametocyte prevalence (60%-68% by molecular diagnosis) did not differ substantially across time points. In contrast, infectivity to mosquitoes, as assessed in membrane feeding assays, varied greatly. Only 15% of individuals were infective in the dry season, compared to 48% at the beginning of the wet season and 34% at the peak of the wet season (Ouédraogo et al., 2016).

To understand the patterns in infectivity across seasons in areas of varied transmission intensity, community surveys involving 1216 observations were carried out in regions of varied transmission intensities, Burkina Faso (high), Mbita, Kenya (moderate) and Kilifi, Kenya (low) to determine the infectiousness of mosquitoes across wet and dry seasons. *P. falciparum* parasite positivity by qPCR for Burkina Faso wet vs

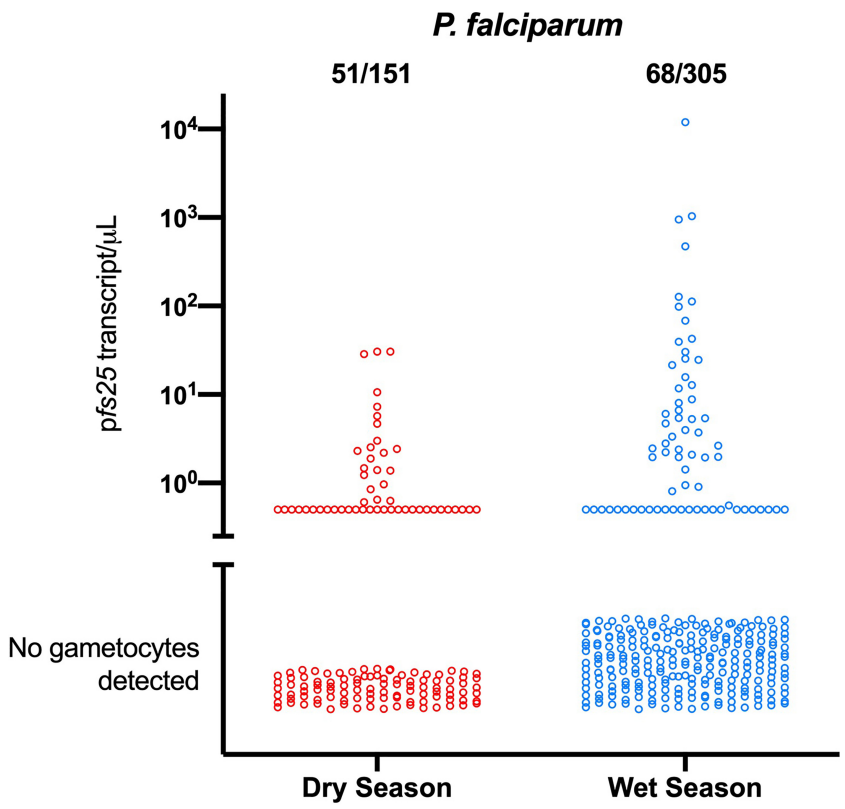

FIGURE 2 | Differences in the proportion of *P. falciparum* gametocyte-positive infections and gametocyte densities between the dry and the wet season in western Kenya (Oduma et al., 2021). In the wet season, fewer infections carried gametocytes, yet mean gametocyte densities were over 3-fold higher. Mean parasite densities did not change, indicating an increased investment in transmission.

dry was 83.6%, 50.0%, and Kilifi wet vs dry was 37.4% and 38.4%, and by microscopy for Mbita wet vs dry was 25.7% vs 28.2%. Across Burkina Faso and Kilifi sites, *P. falciparum* gametocyte carriers and densities by molecular methods were significantly higher in the wet season. To assess the infectivity of study participants, 1209 mosquito feeding assays were performed. A total of 39/1209 (3.2%) of individuals infected at least one mosquito. Proportion of mosquitoes infected per season across the sites did not follow a pattern i.e., Kilifi; dry 0/3046 (0%) vs wet 4/7716 (0.1%), Mbita; dry 28/7071 (0.4%) vs wet 5/6842 (0.1%), Burkina Faso; dry 110/17231 (0.6%), vs wet 121/7749 (1.6%) (Gonçalves et al., 2017).

In Cambodia, asymptomatic individuals were followed in dry and wet seasons to determine infectivity to *An. dirus* mosquito. The study involved 32 and 29 participants for dry and wet seasons respectively. *P. falciparum* gametocyte positivity by RT-PCR was higher in dry season whereas infectivity was relatively high in rainy season. Gametocyte positivity was 71.7% (43/60) and 49% (27/55) among *P. falciparum* infections in the dry and wet seasons respectively. Infectivity among fed *An. dirus* mosquitoes were 2.2% (2/91) and 3.6% (6/168) in dry and rainy seasons respectively (Vantaux et al., 2018).

CONCLUDING REMARKS

An increasing body of research measuring the gametocyte conversion rate, gametocyte densities, and infectivity indicates that *P. falciparum* and *P. vivax* are able to adapt their investment in transmission in response to seasonality and transmission intensity in a site.

Across seasons, *Plasmodium falciparum* is able to increase gametocyte densities and infectivity when transmission increases and vectors are present. This strategy prevents the parasite from spending resources on gametocytes production when the chances of onward transmission is low. Likely, it also benefits the parasite as little or no natural immunity to sexual stages is acquired during the transmission-free season (Kengne-Ouafo et al., 2019). Naturally acquired immunity against gametocytes reduces infectivity. Such immunity is likely short-lived, i.e. the result of recent exposure rather than cumulative exposure (Ouédraogo et al., 2011). Absence of acquired immunity at the start of the transmission season likely results in higher infection success in mosquitos. The stimuli that causes this adjustment is poorly understood. *P. falciparum* parasitized red blood cell - derived microvesicles promote sexual differentiation (Mantel et al., 2013; Regev-Rudzki et al., 2013), however little is known about sensing and signaling pathways and factors that trigger this process. Similarly, there is evidence that the density of uninfected mosquito bites increases in at the start of transmission season (Paul et al., 2004). However, it is not known whether parasites sense abundance of the uninfected mosquito bites at the start of transmission season or they might sense physiological factors of the host body that change in response to seasonality. Increase in transmissibility in the wet season strengthens the rationale for control activities that are adjusted to seasonality, such as seasonal chemoprevention (Konaté et al., 2020; Tchouatieu et al., 2020).

An opposite effect is observed when transmission levels decrease across multiple years, or when parasite population are compared between sites of high and low transmission. The proportion of gametocytes among all *P. falciparum* and *P. vivax* parasites, and infectivity increase when transmission is lower. These long-term changes, which are reflected in the genome, increases the *Plasmodium* parasite's chances for onward transmission even when few vectors are present.

Numerous questions on stimuli affecting the gametocyte conversion rate remain to be answered. Evidence whether the conversion rate is influenced by asexual parasitemia is conflicting. While it was observed in some field and laboratory studies (Carter and Miller, 1979; Schneider et al., 2018; Usui et al., 2019), it was not seen in large population based surveys (Koepfli et al., 2021). It is not known whether presence of mature gametocytes impact on conversion rate. Little is known about the gametocyte epidemiology of other *Plasmodium* sp. infecting humans, i.e., *P. malariae*, *P. ovale*, and *P. knowlesi*, which is emerging in parts of south-east Asia. It remains to be shown whether coinfection of any of these species with *P. falciparum* results in altered gametocyte densities of either species.

Few studies have been conducted on *P. vivax* transmission epidemiology. It is not clear whether observed differences between *P. falciparum* and *P. vivax*, such as different responses to malaria control efforts on Cambodia (Maude et al., 2014), point to different transmission strategies of the two species. A hallmark of *P. vivax* biology is the formation of dormant liver stages that can result in relapsing blood-stage infections weeks to months after the initial infection (Markus, 2012). Relapses result in gametocytemia and thus renewed possibilities for onward transmission (Wampfler et al., 2017). An analysis of historical *P. vivax* data from Finland indicated that *P. vivax* relapses might be triggered by mosquito bites, and thus presence of gametocytes and vectors coincides (Huldén et al., 2008). In how far *P. vivax* is able to time the occurrence of relapses to increase transmission success, and whether such a process is employed as alternative to adjusting the gametocyte conversion rate, is not known.

Increased infectivity of residual infections in populations where transmission has been greatly reduced might be a threat to malaria elimination. Higher infectivity of subpatent infections will reduce the effectiveness of control activities such as reactive case detection (Stuck et al., 2020) or mass screen and treat (Kosasih et al., 2021), which typically rely on rapid diagnostic test or microscopy for diagnosis. This process might be balanced by a higher proportion of all gametocyte carriers being microscopy positive in very low transmission settings (Koepfli et al., 2021). In conclusion, understanding the investment in transmission of parasite populations in different settings can help in informing the design of effective malaria control and elimination strategies.

AUTHOR CONTRIBUTIONS

COO and CK performed the search for relevant literature, read manuscripts, and wrote the final manuscript. All authors contributed to the article and approved the submitted version.

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Infectivity of Symptomatic Malaria Patients to *Anopheles farauti* Colony Mosquitoes in Papua New Guinea

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Plasmodium transmission from humans to mosquitoes is an understudied bottleneck in the transmission of malaria. Direct membrane feeding assays (DMFA) allow detailed malaria transmission studies from humans to mosquitoes. Especially for *Plasmodium vivax*, which cannot be cultured long-term under laboratory conditions, implementation of DMFAs requires proximity to *P. vivax* endemic areas. In this study, we investigated the infectivity of symptomatic *Plasmodium* infections to *Anopheles farauti* colony mosquitoes in Papua New Guinea (PNG). A total of 182 DMFAs were performed with venous blood collected from rapid diagnostic test (RDT) positive symptomatic malaria patients and subsequently analysed by light microscopy and quantitative real time polymerase chain reaction (qPCR). DMFAs resulted in mosquito infections in 20.9% (38/182) of cases. By light microscopy and qPCR, 10 – 11% of *P. falciparum* and 32 – 44% of *P. vivax* positive individuals infected *An. farauti*. Fifty-eight percent of *P. vivax* and 15% of *P. falciparum* gametocytaemic infections infected *An farauti*.

Keywords: direct membrane feeding assay, *Plasmodium vivax*, *Plasmodium falciparum*, Papua New Guinea, *Anopheles farauti*, mosquitoes

Abbreviations: DMFA, Direct membrane feeding assays; *P.vivax*, *Plasmodium vivax*; *P. falciparum*, *Plasmodium falciparum*; *An. Farauti*, *Anopheles farauti*; RDT, Rapid diagnostic kits; HRP2, Histidine rich protein 2; pLDH, Parasite lactate dehydrogenase; qPCR, Quantitative real time polymerase chain reaction assay.

INTRODUCTION

Transmission between the human host and the mosquito vector is a crucial step in the malaria parasite life cycle. It represents a bottleneck where parasite numbers shrink from millions in the human body to less than a hundred in the mosquito vector (Smith et al., 2014). Transmission through the mosquito is thus vulnerable to interruption and is a key focus of malaria research (Churcher et al., 2015; Sauerwein and Bousema, 2015), with research tools including membrane feeding assays designed to explore this transitioning phase of the parasite. DMFAs were initially developed by Rutledge and colleagues in 1964 in which malaria parasites were exposed to mosquitoes *via* a membrane feeding apparatus (Rutledge et al., 1964).

Direct Membrane Feeding Assays provide a means to investigate the still poorly understood process of human to mosquito transmission and the resulting mosquito infection. For example, DMFAs can be used to study the infectiousness of different human malaria reservoirs, and estimate their contribution towards transmission (Graves et al., 1988; Diallo et al., 2008). This can include symptomatic, patent infections as in the present study and asymptomatic, often low-density infections (Kiattibutr et al., 2017). In addition, DMFAs can be used to study the effect of drugs, vaccine candidates and immune factors on the development of the mosquito stages of the *Plasmodium* parasites (Bousema et al., 2012; Delves et al., 2012; Sattabongkot et al., 2015; Vallejo et al., 2016). Also, DMFAs provide an opportunity for circumventing some of the operational and ethical complicating factors associated with feeding mosquitoes directly on the skin of malaria infected individuals. Finally, there is evidence that there is no clustering of gametocytes in the skin as initially perceived thus making DMFAs a reliable tool for infection studies (Meibalan et al., 2019; Talman et al., 2020).

Despite these advantages, DMFAs are resource intensive, require an insectary and rely on stringent logistics for sample collection, handling, rapid transportation and processing as it has been shown that the time between blood collection and performance of the DMFA can impact assay outcome, most likely due to premature gametocyte activation (Churcher et al., 2012; Sattabongkot et al., 2015; Soumare et al., 2021). As a further complication, conducting DMFAs with *P. vivax* requires proximity to endemic areas in order to access infected samples as continuous culture of this parasite species remains elusive (Roobsoong et al., 2015). Papua New Guinea (PNG) is amongst the countries with the highest *P. vivax* burden in the world, thus *P. vivax* is a research priority for the country and infected blood samples can still easily be obtained (Cattani et al., 1986; Müller et al., 2003; Howes et al., 2016; World Health Organization, 2019; World Health Organization, 2020). Establishing DMFAs with *P. vivax* provides a tool to study *P. vivax* transmission that is of potentially global relevance.

DMFAs were performed in PNG previously in 1983 - 1985 in village-based malaria surveys, prior to diagnosis and on known gametocyte carriers in clinical outpatient populations in Madang and Goroka (Graves et al., 1988). In the present study, we investigated the infectivity of blood samples obtained from symptomatic, rapid diagnostic test (RDT)-positive individuals to *Anopheles farauti* colony mosquitoes.

MATERIALS AND METHODS

Sample Collection

This study was conducted at the PNG Institute of Medical Research (PNGIMR) in Madang Province, PNG, between May 2014 and November 2018. Study participants were recruited from Madang Town Clinic and Yagaum Rural Health Centre. Ethical approval was received from the PNGIMR Institutional Review Board (IRB #1516) and the PNG Medical Research Advisory Committee (MRAC #16.01). Written informed consent was received from all individuals enrolled in the study. Individuals presenting with malaria symptoms were tested with a malaria rapid diagnostic tests (RDT). In the present study, CareStart Malaria Pf/PAN (HRP2/pLDH) Ag Combo RDTs kits (Access Bio, Cat No. RMRM-02571CB) were used. From RDT-positive individuals venous blood samples (3 - 5 mL) were collected in vacutainers which contain spray-coated lithium heparin (BD, North Ryde, NSW, Australia) and immediately stored in a beverage cooler flask (Coleman Company Inc, Kansas, USA) filled with warm water (~37.0°C, measured by a digital thermometer attached to the flask). We also measured their Hemoglobin level using a HemoCue machine (HemoCue ®, Mt Waverley, VIC, Australia), their temperature using a digital thermometer and their weight using a bathroom scale.

In the present study, the time between sampling and feeding was approximately 20 - 30 min for samples collected at Yagaum clinic, located in a 10 min walking distance from the insectary. Transport of blood samples collected in Madang town clinic took about 1.5 - 2 h and involved a 30 - 40 min drive.

Mosquito Colony Maintenance, Membrane Feeding Assays, and Mosquito Dissection

The present study used an *An. farauti sensu stricto* colony, which was first adapted in Rabaul, East New Britain province of PNG in 1968. In 1984 females from Agan village, Madang were added to the Rabaul colony in an attempt to back cross. The colony was subsequently used in several studies (Sweeney, 1987; Graves et al., 1988; Beebe et al., 2000; Collins et al., 2002). The colony was maintained using established methods (Nace et al., 2004). To conduct DMFAs, 3 - 5 day old female mosquitoes were put into paper cups (50 -100 per cup). One mL of human blood sample was inserted into the water jacketed glass feeder where we initially exposed up to 400 mosquitoes with 100 mosquitoes per cup (X 4 cups) and then changed with up to 200 with 50 mosquitoes per cup (X 4 cups) as it was less crowded and the feeding rate was much improved (Timinao et al., 2021).

The feeding cups and water jacketed glass feeder were set up in the laboratory prior to the arrival of blood samples to minimise the time between blood sample collection and direct membrane feeding. The light in the insectary was dimmed and the glass feeder with the cups were covered with a dark cloth for the period of feeding. After ~15 - 20 min, the cups of *An. farauti*

were removed from under the glass feeders and any unfed mosquitoes were removed. The cups containing the fully fed mosquitoes were kept for 7 - 9 days before dissecting for oocysts (Ouedraogo et al., 2013; Sattabongkot et al., 2015). Dissection was performed as described elsewhere (Ouédraogo et al., 2013). Briefly, mosquito guts were stained with 0.2% mercurochrome for 10 - 15 min and oocysts were counted under a light microscope at 10 X magnification. Oocysts per midgut were counted once by an experienced microscopist.

Light Microscopy and PCR Detection of Malaria Parasites

Retrospective diagnosis of the malaria parasites was performed by light microscopy and quantitative real-time PCR (qPCR). Thick and thin blood films were prepared using standard WHO methodology. The blood films were stained with 4% Giemsa stain for 30 min (World Health Organization, 2010). Slides were read according to WHO standards and by WHO certified microscopists. Parasite density was calculated by multiplying the parasite count/200 WBC count (or 500 WBC count if the parasite count is <100) by 8000 leukocytes (World Health Organization, 2010). The final parasite density was calculated by taking the geometric mean of the densities obtained from reads by two expert microscopists. Discrepancies in the presence or absence of parasites, parasite density (i.e. if they differed by a factor of 10) and parasite species between the two microscopists was resolved by a third expert microscopist. DNA extraction was performed on 250μL of red blood cell pellets using Favogen DNA extraction kits (Favogen Biotech Corp, Ping Tung, Taiwan) and performed according to the protocol for extraction of genomic DNA from blood. Following DNA extraction, a qPCR assay was performed to quantify the infection and determined the parasite species as described elsewhere (Wampfler et al., 2013). Briefly, this is a probe based qPCR assay where a conserved region of the 18SrRNA gene was amplified for both *P. falciparum* and *P. vivax*.

Statistical Analyses

Prism 6.01 (GraphPad Software, La Jolla, CA USA) and Stata 13 (StataCorp, College Station, TX, USA) were used to analyse data. To compare proportions, two-sample tests of proportions were used. To test the influence of a continuous variable (such as parasite density) on a binary outcome variable (such as DMFA success rate), logistic regression was used. To test the association between two continuous variables such as infection rate in the successfully infected mosquitoes versus gametocyte density we used non-parametric correlation analysis (Spearman's rank correlation).

RESULTS

Study Population

Selection of patients relied on RDT diagnosis. Subsequent light microscopy examinations of the corresponding blood slides and molecular diagnosis by qPCR were conducted for 182 RDT-positive participants. A total of 45 patients were recruited from Madang town clinic while 137 were recruited from Yagaum clinic. **Table 1** shows the characteristics of the study population and **Table 2** shows the results from RDT, light microscopy examination and molecular diagnosis by qPCR.

TABLE 1 | Characteristics of the study population.

Demography	Median (range) or n/N (%)
Age in years (n=182[a])	17 (5-55)
Female (n=182)	91/182 (50.0%)
Weight, kg, (n=175[b])	47 (14-96)
Hemoglobin, g/dl, (n=118[b])	9.1 (4.2-13.7)
Temperature, °C, (n=161[b])	36.6 (34.1-40)
Fever, >37.5°C, (n=161)	48/161 (29.8%)

[a]*Eight individuals with unknown age so were considered as adults.*
[b]*These data were not collected from all 182 patients.*
Values are presented as proportions (n/N) and percentage or median and range.

Malaria Diagnosis

The largest proportion of individuals (49.5%; 95% CI 41.97 - 56.95%) was RDT positive for both, HRP2 and pLDH tests while 30.2% (95% CI 23.65 - 37.45%) and 20.3% (95% CI 14.74 - 26.92%) of patients were positive only for HRP2 or pLDH-based tests, respectively. Light microscopy revealed that the largest proportion of symptomatic patients in this study population were infected with *P. falciparum* (47.8%; 95% CI 40.90 - 55.86%) followed by *P. vivax* (28.6%; 95% CI 22.13 - 35.72). Median (range) parasite density was 6423 (110 – 51,040) parasites/μL for *P. falciparum* and 4240 (136–32,480) parasites/μL for *P. vivax*. There were 3 mixed infections (1.6%; 95% CI 0.3 - 4.74%) containing both, *P. falciparum* and *P. vivax*. The qPCR results revealed a slightly higher proportion of *P. falciparum* infections (40%; 95% CI 36.62 - 51.49%) than *P. vivax* infections (30.2%; 95% CI 23.65 - 37.45%). A higher proportion of the samples were diagnosed as mixed infections by qPCR as compared to microscopy (11% vs 1.6%). Over all we observed a higher proportion of patients who were diagnosed as *P. falciparum* positive by microscopy and qPCR as compared to RDT diagnosis (**Table 2**).

A total of 154/182 (85%) of the samples were concordant between qPCR and microscopy diagnosis. It was also observed that 19/182 (10.4%) patients were negative by microscopy but were positive by qPCR for malaria parasites while 9/182 (5%) were positive by microscopy but negative for qPCR (**Table 3**). A sensitivity of 94% was observed for qPCR diagnosis with a specificity of 49%. The positive predictive value (PPV) of 88% and a negative predictive value (NPV) of 67% was observed for qPCR. We also observed that there was no correlation between the microscopy diagnosis and qPCR for the following groups; *P. vivax, P. vivax* with gametocytes and *P. falciparum* with gametocytes. However, there was a significant but weak correlation observed with *P. falciparum* (Spearman's rank correlation coefficient R=0.4, p<0.001).

Mosquito Infection

Overall, 38/182 (20.9%) of blood samples in DMFAs infected mosquitoes with 36/38 (94.7%) of the patients being recruited

TABLE 2 | RDT, Microscopy and qPCR diagnosis of malaria parasites.

Diagnosis		n	n/N (%)	95% CI
RDT	HRP2	55	30.2	23.65 - 37.45
	pLDH	37	20.3	14.74 - 26.92
	HRP2 & pLDH	90	49.5	41.97 - 56.95
Microscopy	*P. falciparum* asexual only	68	37.4	23.65 - 37.45
	P. falciparum asexual with gametocytes	20	11.0	6.84 - 16.46
	P. vivax asexual only	28	15.4	10.47 - 21.46
	P. vivax asexual with gametocytes	24	13.2	8.63 - 18.98
	P.falciparum with gametocytes & *P.vivax* with gametocytes	2	1.1	0.13 - 3.91
	P.falciparum asexual & *P.vivax* with gametocytes	1	0.5	0.01 - 3.02
	Microscopy negative	37	21.4	15.70 - 28.11
qPCR	*P. falciparum*	80	44.0	36.62 - 51.49
	P. vivax	55	30.2	23.65 - 37.45
	P.falciparum & *P.vivax* mix	20	11.0	6.84 - 16.46
	PCR negative	27	14.8	10.01 - 20.85

Results of the diagnosis by RDT, microscopy and qPCR, N=182 samples in each case.

from Yagaum clinic while the remaining 2/38 (5.3%) were Madang town clinic. **Figure 1** shows an example of an *An. farauti* midgut infected with *P. vivax* oocysts 7 days post infection.

Although not significant we did observe a higher proportion of infections by individuals diagnosed with RDT as pLDH positive than those diagnosed as HRP2 positive (35.1% vs 27.3%, p=0.43) (**Table 4**). Interestingly, *P. vivax* infections diagnosed by light microscopy were significantly more infectious to mosquitoes compared to *P. falciparum* infections (44.2% vs. 11.4%, p<0.01). Similar observations were made with qPCR diagnosis (43.6% vs 10%, p<0.001). Within the *P. vivax* samples, a higher proportion of blood samples were infectious to mosquitoes when *P. vivax* gametocytes were detected by microscopy (58.3%). We noted that 32% (9/28) and 10.3% (7/68) of the *P. vivax* and *P. falciparum* infections that infected mosquitoes were gametocytaemic by microscopy. In addition, all the mixed infections (3/3) by microscopy gave rise to mosquito infections.

There was a weak correlation between the proportion of infected mosquitoes and *P. vivax* density by microscopy with the correlation approaching significance (p=0.08, Spearman's rank correlation coefficient R = 0.4) as shown in **Figure 2A**. The proportion of infected mosquitoes was significantly correlated with *P. vivax* gametocyte density (p<0.05, Spearman's rank correlation coefficient R=0.6) as shown in **Figure 2B**. However, the considerable scatter and correlation coefficient of R=0.6 indicated that the correlation was not very strong. There was no correlation between the mosquito infection rate and the copy numbers of *P. falciparum* or *P. vivax* by qPCR.

Infection success, i.e., DMFAs resulting in at least 1 infected mosquito, was not significantly correlated with parasite or gametocyte density when tested using logistic regression in any of these groups; *P. vivax*, *P. falciparum*, *P vivax* with gametocytes.

We observed moderate and significant correlations between the number of oocysts per infected mosquito midgut and the proportion of infected mosquitoes per DMFA according to microscopy diagnosis for the following; *P. vivax* (Spearman's rank correlation coefficient R=0.7, p<0.0001), *P. vivax* with gametocytes (Spearman's rank correlation coefficient R=0.7, p<0.01), and *P. falciparum* (Spearman's rank correlation coefficient R= 0.7, p<0.05) as shown in **Figures 3A–C**. We also observed a moderate and significant correlation between oocysts per infected mosquitoes and mosquito infection by qPCR for *P. vivax* according to qPCR diagnosis (R=0.7, p<0.001) as shown in **Figure 3D**. There was no correlation observed between the proportion of infected mosquitoes and the copy numbers of *P. vivax* or *P. falciparum* by qPCR.

DISCUSSION

Currently the limitation with doing research with *P. vivax* is it is still difficult to maintain a continuous culture of *P. vivax*, which necessitates access to naturally acquired infections in field settings, often associated with additional cost and operational constraints. As such, a reliable *P. vivax* DMFA setup in an endemic setting can be of great value.

In the present study, we investigated the infectiousness of symptomatic, RDT-positive malaria cases obtained from local health facilities. In a resource constrained situation where diagnosis by microscopy is not readily available and where the primary diagnosis of malaria is performed by RDT, it is

TABLE 3 | Comparison of malaria parasite detection in patients' blood by microscopic examination and qPCR.

		Microscopy (Gold standard)		
	Test Result	**+**	**–**	**Total**
qPCR	+	136	19	155 (85%)
	–	9	18	27 (15%)
	Total	145 (80%)	37 (20%)	182 (100%)

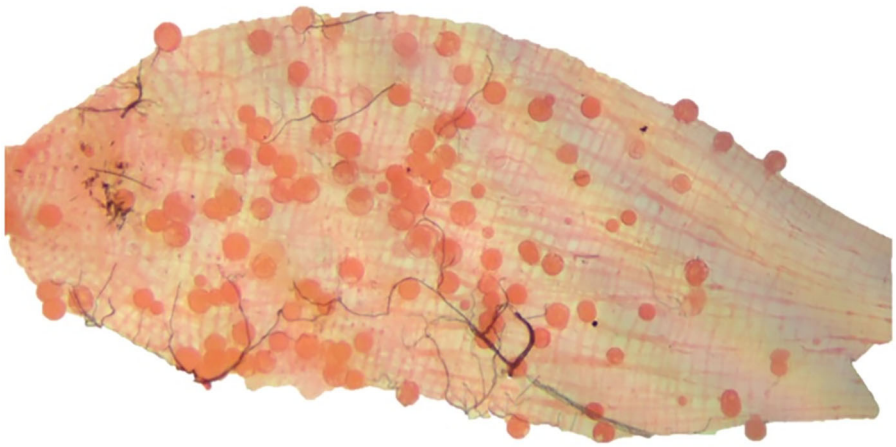

FIGURE 1 | *P. vivax* infected midgut from *An. farauti* mosquito dissected in the present study. The image was taken on a Zeiss Primostar microscope equipped with an Axiocam 105 Color camera (Carl Zeiss Pty. Ltd.) at 10x magnification. The image was then edited using PowerPoint, Microsoft office 2010 and Adobe Photoshop CS6.

TABLE 4 | Mean oocyst counts from DMFAs in *An. farauti* according to RDT, microscopy and qPCR.

RDT, microscopy & qPCR results	Proportion DMFAs resulting in mosquito infection		Proportion of mosquitoes infected*		Oocyst number
	n/N	% (95% CI)	n/N	% (95% CI)	average (range)
RDT					
HRP2	15/55	27.3 (15.5 - 39.1)[a]	564/966	58.4 (55.2 - 61.5)	6 (1-106)
pLDH	13/37	35.1 (19.7 - 50.5)[b]	349/863	40.4 (37.2 - 43.8)	27 (1-534)
HRP2 & pLDH	10/90	11.1 (4.6 - 17.6)[c]	60/415	14.5 (11.2 - 18.2)	3 (1-17)
Microscopy					
Pf asexual only	7/68	10.3 (3.1 - 17.6)[d]	66/376	17.6 (13.8 - 21.8)	5 (1-16)
Pf. + gametocytes	3/20	15 (0 - 30.6[§])	47/94	50.0 (39.5 - 60.5)	3 (1-9)
Pv. asexual only	9/28	32.1 (14.8 - 49.3)[e]	218/627	34.8 (31 - 38.6)	9 (1-93)
Pv. + gametocytes	14/24	58.3 (38.6 - 78)[f]	424/749	55.9 (52.8 - 60.2)	19 (1-534)
Pf. + gametocytes & *Pv* + gametocytes	2/2	100 (NA)	13/69	18.8 (10.4 - 30.1)	3 (1-13)
Pf. asexual only & *Pv* gametocytes	1/1	100 (NA)	83/89	93.3 (85.9 - 97.5)	7 (1-36)
Microscopy Negative	2/39	5.1 (0 - 12[§])	13/38	34.2 (19.6 - 51.4)	9 (1-29)
qPCR					
P. falciparum	8/80	10 (3.4 - 16.6)	135/481	28 (24.1 - 32.3)	3 (1-43)
Pv.	24/55	43.6 (30.5 - 56.7)	735/1501	49 (46.4 - 51.5)	12 (1-534)
P. falciaprum & P. vivax	4/20	20 (2.5 - 37.5)	96/248	38.7 (32.6 - 45.1)	8 (1-106)
qPCR Negative	2/27	7.4 (0 - 17.3)[§]	7/14	50.0 (23.0 – 77)	7 (1-24)

Results of the diagnosis by RDT, microscopy and qPCR, N =182 while n = 38 in the successful infections.

**only infected mosquitoes were considered (i.e., uninfected mosquitoes were not included into this calculation); significant differences were observed in the proportions [a] vs. [c]; [b] vs. [c], [d] vs. [e] and [d] vs. [f]. No significant difference was observed between [a] vs. [b], p=0.43.*

[§]95% confidence interval includes negative values.

All samples were collected from symptomatic RDT positive patients. Values are presented either as proportion (n/N) and percent, or as average and minimum to maximum range.

Pf., P. falciparum; Pv., P. vivax.

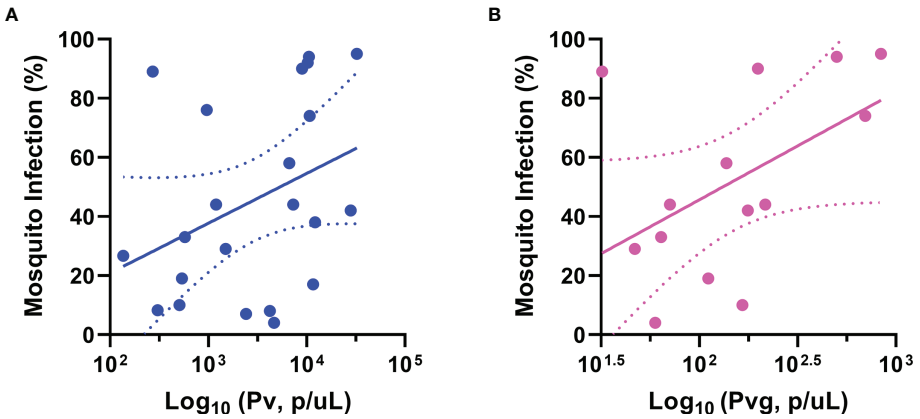

FIGURE 2 | Correlation between mosquito infection rate and parasite burden of infected humans. Correlation between mosquito infection and **(A)** *P. vivax* parasites (R= 0.4, p=0.08), **(B)** *P. vivax* gametocytes (R= 0.6, p=0.04). The trend lines are linear regression while the area between the dotted lines represents the 95% confidence interval. Pv, *Plasmodium vivax*; Pvg, *P. vivax* gametocytes. Each dot represents a mosquito that was infected with 1 or more oocyst. **(A)** has 22 while **(B)** has 14 successful infections.

important to assess which RDT result will most likely lead to a mosquito infection. As commonly known, RDT results are not reliable in distinguishing between *Plasmodium* species in co-endemic settings, however, the present study shows that they can be used to prioritise samples selected for DMFAs to maximise the probability of a specific species being present in the sample and to increase infection success (**Table 2**) (World Health Organization, 2006). We observed that in the group of samples positive for only pLDH with the CareStart RDT the proportion of successful DMFAs was highest (35.1%) compared to HRP2 (27.3%) or when positive for both antigens (11.1%). This difference in proportions was statistically significant between pLDH and both antigens ($p<0.01$) but not between pLDH and HRP2 ($p=0.43$). In PNG where both *P. falciparum* and *P. vivax* are present in roughly equal proportions, *P. vivax* infections were more likely when the RDT is positive for only the pLDH antigen (World Health Organization, 2017). We therefore decided that by selecting samples only positive for pLDH over HRP2 (or both antigens) the likelihood of the sample being *P. vivax* would be significantly increased and DMFA success can be increased up to 3-fold. There is a sound biological explanation as to why acute *P. vivax* infections result in mosquito infections more frequently. It has been shown that *P. vivax* gametocytes develop faster, and are present and infectious at the onset of an infection while *P. falciparum* gametocytes can take 10 days to mature (Bousema and Drakeley, 2011). Consequently, lower infectiousness in symptomatic *P. falciparum* patients as compared to *P. vivax* patients is expected since people are likely to seek treatment before *P. falciparum* gametocytes have matured (Kiattibutr et al., 2017). Furthermore, HRP2 based RDTs can remain positive for 35-42 days after treatment and clearance of parasitaemia, while for pLDH it takes only 2 days before the antigen is cleared from circulation giving a more reliable result (Grandesso et al., 2016).

We observed that the proportion of samples that infect mosquitoes was higher for *P. vivax* (44.2%) compared to *P. falciparum* (11.4%) according to microscopy. Interestingly, we observed a higher mosquito infection rate (58.3%) for samples with *P. vivax* gametocytes detectable by light microscopy while the mosquito infection rate with *P. falciparum* gametocytes was low (15%). Although our findings are in contrast to what was observed previously by Graves and colleagues in *An. farauti* where they showed a 37.5% (6/16) infectivity with *P. vivax*, 18.8% (3/16) infectivity in *P. vivax* with gametocytes and a 48.1% (13/27) infectivity in *P. falciparum* with gametocytes, this may be due to the difference in sample sizes used (Graves et al., 1988). Our results show that our DMFA with *P. vivax* is about 4 times more successful than with *P. falciparum* especially when considering samples with gametocytes. Although we are uncertain as to why we observed low *P. falciparum* infections a possible explanation that we did not evaluate in this study is that immunity-related factors are responsible. This could be further studied by comparing, in parallel, DMFAs conducted with autologous plasma (i.e., replacement of patient's plasma with the individual's own plasma), whole blood (directly added to the feeder) and malaria-naïve plasma (i.e., replacement of patient plasma with plasma from a *P. falciparum* naive donor). Furthermore, a possible explanation is that this strain of mosquitoes may have become refractory to wild-type *P. falciparum* infection. This was observed with cultured gametocytes of *P. falciparum* which were fed to this strain of *An. farauti* mosquitoes *via* standard membrane feeding which resulted in an extremely low mosquito infection rate (Smith et al., 2014). This indicated that the *An. farauti* strain was refractory to cultured gametocytes and that could also be the case with wild-type parasites as well. Interestingly, our observations that this does not apply to *P. vivax* may be the basis for further studies into species-specific mechanisms of mosquito infection.

In the present study, we observed an 85% concordance between the microscopy diagnosis and qPCR diagnosis. We observed a 94% sensitivity and a 49% specificity when

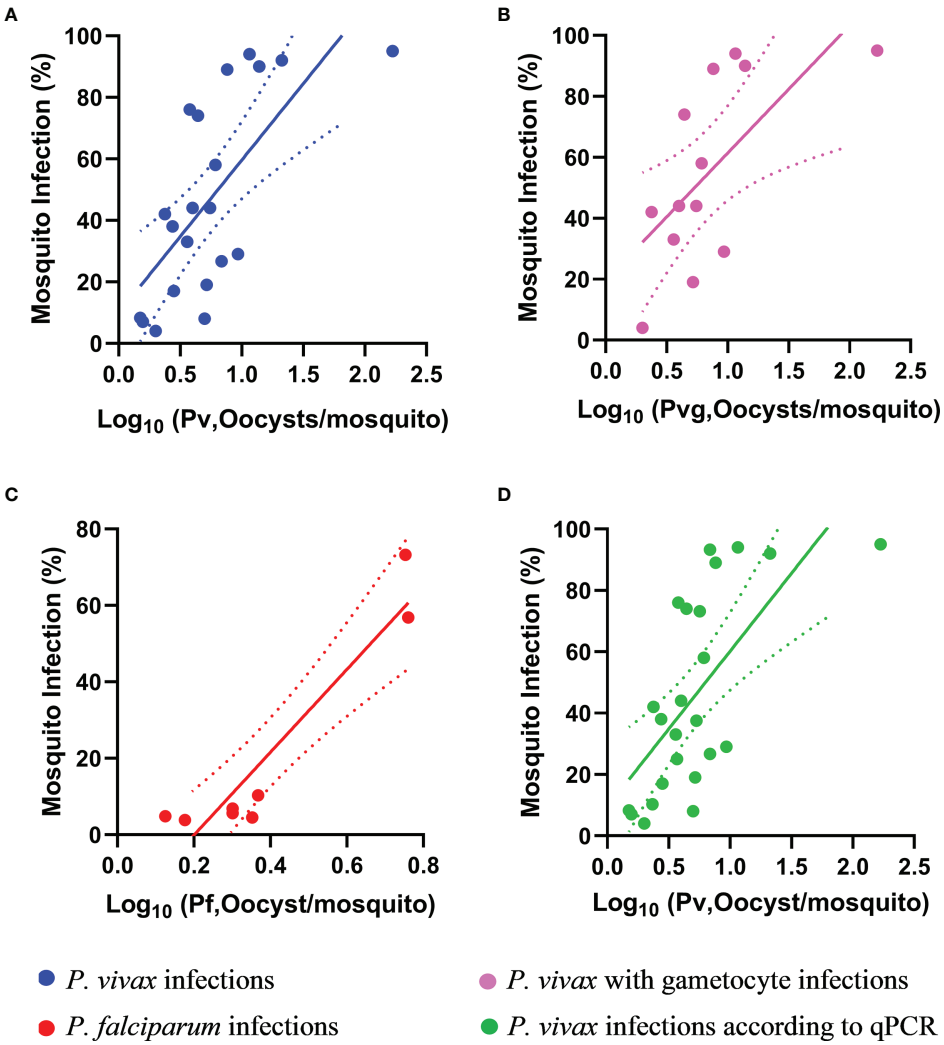

FIGURE 3 | Correlation between proportion of infected mosquitoes and the mean oocyst number per infected mosquito. The oocysts per infected mosquito for **(A)** *P. vivax* (R = 0.7, p < 0.001), **(B)** *P. vivax* with gametocytes (R = 0.7, p < 0.01), **(C)** *P. falciparum* (R = 0.8, p < 0.05) and **(D)** *P. vivax* by qPCR (R = 0.7, p < 0.001). The trend lines are liner regression while the area between the dotted lines represents the 95% confidence interval. Pv, *Plasmodium vivax*; Pvg, *P. vivax* gametocytes; Pf, *P. falciparum*.

comparing qPCR with light microscopy as the reference method. This is a result of the lower limit of detection of the qPCR method, which is able to detect many more infections as compared to light microscopy. As qPCR is able to detect these sub-microscopic infections, the proportion of false positive is overestimated when compared to light microscopy leading to an apparently low specificity. The possibility of an infection (or no infection) by microscopy being confirmed by qPCR is expressed by a moderate PPV and NPV (88% and 67%). We note that the lack of having microscopy diagnosis being done prior to bleeding was a limitation in this study and light microscopy results were only obtained retrospectively by highly trained microscopists. We found that species and parasite stage determination by light microscopy was a very good predictor of infection success, as *P. vivax* with gametocyte infections resulted in approximately 4-fold increased infection success in the mosquitoes as compared to *P. falciparum*. Based on our results we estimate that light microscopy diagnosis before bleeding would enable a further increase of DMFA success rate with *P. vivax* to around 60% if suitable *P. vivax* samples (those with gametocytes by light microscopy) were selected. Similar infectivity rates (45-60%) were measured in *Anopheles aquasalis, Anopheles albitarsis* in Brazilian Amazon, *Anopheles albamanus* in Colombia, *An. dirus* in Thailand and *Anopheles arabiensis* in Ethiopia (Sattabongkot et al., 2003; Solarte et al., 2011; Rios-Velásquez et al., 2013; Vallejo et al., 2016; Tadesse et al., 2018). We did also observe a significant but moderate correlation between *P. vivax* gametocytes and mosquito infection (**Figure 2B**). Other studies observed similar but often stronger positive associations between *P. vivax* gametocyte densities and the proportion of infected

mosquitoes in *An. dirus* in Thailand and *An. arabiensis* in Ethiopia (Kiattibutr et al., 2017; Tadesse et al., 2018). However, there are other studies which describe the relationship between *P. vivax* gametocytaemia and mosquito infection as weak with *An. dirus* in Thailand (Sattabongkot et al., 1991; Sattabongkot et al., 2003).

The observed correlation between mosquito infection prevalence and oocyst density was moderate but significant for both *P. falciparum* and *P. vivax* (**Figures 3A–D**). That is, the more mosquitoes are infected during a DMFA, the higher the average number of oocysts in the infected mosquitoes. Our findings is in contrast with a previous study where a strong correlation was observed between the mosquito infection rate and the oocyst rates for *An. dirus* with *P. vivax* (Kiattibutr et al., 2017).

We noted that only 2/45 (4.4%) of samples resulted in mosquito infections from DMFA using blood from the Madang Town Clinic while 36/137 (26.2%) of samples from Yagaum clinic infected mosquitoes. The low infection rate from Madang Town Clinic was mainly because most of the samples were without gametocytes especially *P. vivax* gametocytes. Of the 45 samples 3 samples had only *P. falciparum* gametocytes while 2 had only *P. vivax* gametocytes and one with both *P. falciparum* and *P.vivax* gametocytes. Of the 2 samples that led to successful infections, one had only *P. vivax* gametocytes while the other had both *P.falciparum* and *P. vivax* gametocytes. Another factor which could have influenced the infectivity of the mosquitoes but was not investigated here is impact of temperature fluctuations of the thermal flask while transporting it from Madang to the laboratory, and the longer duration between collection of the sample and the DMFA. It has been shown elsewhere that temperature of thermal flask does influence the infectivity of the mosquitoes (Soumare et al., 2021).

This study provides important insights into the infectivity of symptomatic malaria cases to *An. farauti* in PNG. Overall, we show that symptomatic *P. vivax* infections are more likely to be infectious to mosquitoes as compared to symptomatic *P. falciparum* infections. This may be a result of the differences in gametocyte dynamics that exist between *P. falciparum* and *P. vivax*. We have re-established a DMFA set up in PNG, where frequent access to *P. vivax* infections is provided. This could serve as a platform to test potential transmission blocking vaccines and antimalarials, which act on gametocytes or the mosquito developmental stages of *P. vivax*.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Papua New Guinea Medical Research Advisory Council. Written informed consent to participate in this study was provided by the participants or their legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

Designed the study: LR, SK, ML. Conducted the laboratory work: LT, RV, MK, TK, EN. Secured funding: IM, IF, SK. Drafting and preparation of the manuscript: LT, SK. Critically revising the manuscript: SK, RV, MK, TB, LS, IF, LR, CC. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

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Gene Polymorphisms Among *Plasmodium vivax* Geographical Isolates and the Potential as New Biomarkers for Gametocyte Detection

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The unique biological features of *Plasmodium vivax* not only make it difficult to control but also to eliminate. For the transmission of the malaria parasite from infected human to the vector, gametocytes play a major role. The transmission potential of a malarial infection is inferred based on microscopic detection of gametocytes and molecular screening of genes in the female gametocytes. Microscopy-based detection methods could grossly underestimate the reservoirs of infection as gametocytes may occur as submicroscopic or as micro- or macro-gametocytes. The identification of genes that are highly expressed and polymorphic in male and female gametocytes is critical for monitoring changes not only in their relative proportions but also the composition of gametocyte clones contributing to transmission over time. Recent transcriptomic study revealed two distinct clusters of highly correlated genes expressed in the *P. vivax* gametocytes, indicating that the male and female terminal gametocytogeneses are independently regulated. However, the detective power of these genes is unclear. In this study, we compared genetic variations of 15 and 11 genes expressed, respectively, in the female and male gametocytes among *P. vivax* isolates from Southeast Asia, Africa, and South America. Further, we constructed phylogenetic trees to determine the resolution power and clustering patterns of gametocyte clones. As expected, *Pvs*25 (PVP01_0616100) and *Pvs*16 (PVP01_0305600) expressed in the female gametocytes were highly conserved in all geographical isolates. In contrast, genes including 6-cysteine protein *Pvs230* (PVP01_0415800) and upregulated in late gametocytes *ULG8* (PVP01_1452800) expressed in the female gametocytes, as well as two CPW-WPC family proteins

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(PVP01_1215900 and PVP01_1320100) expressed in the male gametocytes indicated considerably high nucleotide and haplotype diversity among isolates. Parasite samples expressed in male and female gametocyte genes were observed in separate phylogenetic clusters and likely represented distinct gametocyte clones. Compared to *Pvs*25, *Pvs230* (PVP01_0415800) and a CPW-WPC family protein (PVP01_0904300) showed higher expression in a subset of Ethiopian *P. vivax* samples. Thus, *Pvs230*, *ULG8*, and CPW-WPC family proteins including PVP01_0904300, PVP01_1215900, and PVP01_1320100 could potentially be used as novel biomarkers for detecting both sexes of *P. vivax* gametocytes in low-density infections and estimating transmission reservoirs.

Keywords: *Plasmodium vivax*, gametocyte detection, molecular biomarkers, genetic differentiation, malaria transmission

INTRODUCTION

Plasmodium vivax malaria is a neglected tropical disease, despite being more geographically widespread than other forms of malaria (Organization, 2018) and causes 132–391 million clinical infections each year worldwide (Price et al., 2007). *Plasmodium vivax* infections are most common in Southeast Asia and South America, with the Southeast Asian isolates being the most genetically diverse (Rougeron et al., 2020). Compared to *P. falciparum*, *P. vivax* has a broader temperature tolerance and an earlier onset of gametocyte development, enabling the parasites to spread through diverse climates (Elgoraish et al., 2019) and making them more difficult to control and eliminate (Lo et al., 2017). Although *P. vivax* infection is relatively benign, patients may experience similar clinical symptoms as *P. falciparum*, such as inflammatory responses, fever, and chills (Dayananda et al., 2018). The epidemiology of *P. vivax* malaria is further complicated by the parasite's unique ability to form dormant-stage hypnozoites in the host liver cells, resulting in recurrent relapse infections from weeks or months to years later (White, 2011; Chu and White, 2016). Hypnozoites, which remain arrested in the liver for weeks to years, can be activated to cause new blood-stage infections and these features have substantially impacted progress in malaria control, especially in countries that are approaching elimination by providing parasite reservoirs for transmission at any time (Robinson et al., 2015; Lawpoolsri et al., 2019; Taylor et al., 2019).

A critical stage in *P. vivax* transmission is the development of gametocytes, known as gametocytogenesis, from trophozoites in the erythrocytic cycle. This allows *Plasmodium* species to be taken up by a mosquito vector, undergo sexual reproduction in the mosquito gut, and transmit to a new human host. Indeed, in the mosquito midgut, parasites can differentiate into their sexual forms, the female macrogametes and male microgametes. Gametocyte commitment is largely based on stress factors including high parasitemia, parasite strains, red blood cell density, anemia, drug treatments, and host immune responses (Smalley and Brown, 1981; Trager and Gill, 1992; Gautret et al., 1996; Talman et al., 2004; Drakeley C et al., 2006; Koepfli et al., 2015). Changes in temperature, pH, and host age can also stimulate gametogenesis in the human host (Bousema and Drakeley, 2011). When gametocytes are taken up during a mosquito's blood meal, a number of factors including temperature, oxygen and carbon dioxide concentration, and pH can contribute to the maturation of gametocytes inside the mosquito midgut and salivary glands (Sinden, 1997). Other mosquito-derived factors such as xanthurenic acid can together activate transformation of gametocytes to male microgamete and female macrogamete within 5-10 minutes in the mosquito midgut. Although no clinical symptoms are experienced in the human hosts during gametocytogenesis, this developmental stage is critical for sexual reproduction in the mosquitoes and subsequent development of sporozoites that can infect other new human hosts. There is considerable variation in the development time of gametocytes amongst different human *Plasmodium* species, ranging from 7-10 days after the initial establishment of asexual parasites for *P. falciparum* (Gardiner and Trenholme, 2015) and 7-15 days for *P. vivax* (Bousema and Drakeley, 2011), indicating the need for reliable biomarkers for early-stage gametocyte detection.

A previous study has shown approximately 10% of *P. falciparum* and 60% of *P. vivax* infections have concurrent detectable low-density gametocytemia (Tadesse et al., 2017). Molecular tests for diminutive amounts of gametocytes rely on reverse-transcription polymerase chain reaction (RT-PCR) to amplify RNA transcripts of gametocyte-specifically expressed genes. Quantitative RT-PCR of targeted RNA transcripts reveals high sensitivity in detecting gametocytes of considerably low densities. For example, there are more than 106 copies of 18S rRNA transcripts per cell but only 5 copies of 18S rRNA gene per genome (Nishimoto et al., 2008). The production of high transcript copies in the parasite cells allow for greater detection limits. For *P. vivax*, *Pvs*25 and *Pvs*16, genes specific to the female gametocytes, are the two conventional gene markers for gametocyte detection (Wampfler et al., 2013). One gametocyte roughly corresponds to four *Pvs*25 transcripts per cell (Koepfli et al., 2015), and *Pvs*25 can detect from approximately 0.34 gametocytes per microliter of blood from *P. vivax* patients in Papua New Guinea (Wampfler et al., 2013) to 2 gametocytes per microliter of blood from *P. vivax* patients in Ethiopia (Tadesse et al., 2017). The number of *Pvs*25 gene transcript copies detected by qRT-PCR directly correlated with the number of mature gametocytes as well as the overall parasite densities (Bharti et al., 2006; Bousema and Drakeley, 2011) and

showed a nearly normal distribution with a mean of 1.2×10^7 copies/μL (ranging from 1.1 to 4.8×10^8 copies/μL) blood among symptomatic *P. vivax* patients in northwestern Brazil (Lima et al., 2012). Such low gametocyte densities make them extremely difficult to be detected by microscopy and highlight the importance of sensitive molecular biomarkers in malaria-endemic regions.

Gametocytes are generally detected in ~20% of the infections among adults (Bousema and Drakeley, 2011), but at much higher proportions in children under the age of 12 (Nacher et al., 2004; Olliaro et al., 2016). Yet, gametocytemia in adults is up to 20-fold higher than in children (Dixon et al., 2008; Reece et al., 2009). In areas with low transmission, submicroscopic gametocytes could be hidden reservoirs for parasites with high proportions of infectious gametocytes (Hofmann et al., 2018). In Ethiopia, symptomatic *P. vivax* infections are nearly four times more infectious than asymptomatic ones (Tadesse et al., 2018). A recent study of 26 P. *vivax* samples from Cambodian patients indicated that the expression profile of 21 predicted gametocyte genes were clustered in two distinct groups (Kim et al., 2019). One group includes *Pvs*25, *ULG*8, gametocyte developmental protein 1, guanylate kinase, *HMGB*1, and five CPW-WPC proteins that associate with intracellular trafficking and histone remodeling in the female gametocytes. The other group includes *Pvs*47, *Pvs*48/45. *Hap*2, the gamete egress and sporozoite traversal protein, s16, and three CPW-WPC proteins that associate with microtubular development in the male gametocytes. It remains unclear if these male and female gametocyte genes show higher expression than the conventional marker *Pvs*25 and offer high detectability of total gametocyte densities (i.e., both male and female gametocytes). The detection of total gametocytes allows for robust gametocyte sex-ratio estimates in field studies given their stability under suboptimal conditions (Meerstein-Kessel et al., 2018). Gametocytes in low density infections can initiate transmission at any time, increasing the need to utilize reliable biomarkers for their detection and control. At the genomic level, polymorphisms in gametocyte-specific genes may provide information of parasite reservoirs that are transmitted from infected humans to mosquitoes, offering new insights into transmission bottlenecks in vectors. Genetic polymorphisms in gametocyte genes including *Pvs*25, *Pvs*28, *Pvs*48/45, and *Pvs*230 will also have important implications on their potential use and effectiveness as transmission blocking vaccine (TBV) candidates. Therefore, there are two key objectives in this study. First, genetic variations of 17 and 11 genes that represent female and male gametocytes, respectively, were compared among *P. vivax* isolates from Southeast Asia, East Africa, and South America based on whole genome sequence data. We constructed phylogenetic trees to determine clustering patterns with the goal of identifying novel DNA biomarkers with high differentiative power for gametocyte clones. Second, the expression levels of several female and male gametocytes were compared among a subset of Ethiopian *P. vivax* isolates based on transcriptomic data with the goal of identifying highly sensitive RNA biomarkers for both sexes of *P. vivax* gametocytes.

MATERIALS AND METHODS

Data Collection

Whole blood samples were collected from 22 P. *vivax*-infected patients in Jimma, southwestern Ethiopia between September and November of 2016. We used Lymphoprep/Plasmodpur-based protocol to deplete the white blood cells and enrich the red blood cell pellets prior to DNA and RNA extractions. Genomic DNA was extracted from ~1 mL red blood cell pellets using the Quick-DNA Miniprep Kit (Zymo Research) following the manufacturer's protocols. Only samples with monoclonal infections based on microsatellite genotyping were included for whole genome sequencing. These samples were collected from areas in Southern Ethiopia including Arbaminch, Badowacho, Halaba, and Hawassa (Auburn et al., 2019; Ford et al., 2020). An additional 39 samples of sequence data were obtained as FASTQ files from the European Nucleotide archive (ENA) that represent other East African countries including Uganda, Sudan, and Eritrea (Benavente et al., 2021). Sequence reads were mapped to the P01 reference genome (Auburn et al., 2016) available in Gene DB using BWA-MEMv2 (Li and Durbin, 2010; Langmead and Salzberg, 2012) with default settings. Only reads that were mapped to the reference were included and the quality of each of the aligned maps were assessed using FASTQC. The percentage coverage of the *P. vivax* reads was high for all samples (Ford et al., 2020). To provide a comparison of polymorphisms of our panel of gametocyte genes, we obtained an additional 72 P. *vivax* genomes including 50 genomes from southeast Asia (Cambodia and Thailand) and 22 from South America (Panama and Peru) (Pearson et al., 2016; Buyon et al., 2020). These genomes were obtained as FASTQ files from the ENA and Genbank. These genomes were also aligned to the P01 reference genome using BWA-MEMv2.

SNP Discovery and Gene Diversity Analyses

Potential single nucleotide polymorphisms (SNPs) were identified using the genome analysis toolkit (GATK) version 4 (Van der Auwera and O'Connor, 2020) across all samples using the P01 reference genome. For the variant calling, we filtered the reads with the following scores: QD (quality by depth) less than 2.0, QUAL (read quality) less than 30, SOR (strand odds ratio) greater than 3.0, and MQ (map quality) score less than 40. From the high-quality SNPs, we obtained the consensus sequences of 28 gametocyte genes for further analyses. These 28 gametocyte genes were selected based on a previous study that showed the expression levels of several gametocyte markers were highly correlated with each other and clustered into two distinct groups (Kim et al., 2019). The microtubule-associated proteins including dynein, kinesin, and tubulin as well as few other male gametocyte genes were overrepresented in one cluster, while intracellular trafficking and histone remodeling genes were overrepresented in another. Therefore, in this study, a total of 17 female gametocyte genes including gametocytes ookinete surface protein *Pvs*25 (PVP01_0616100), gametocyte associated protein (PVP01_1403000), 6-cysteine protein *Pvs230* (PVP01_0415800), genes from the CPW-WPC family protein

(PVP01_0820000, PVP01_0904300, PVP01_1003000, and PVP01_1223200), Guanylate kinase *PvGK* (PVP01_0727400), upregulated in late gametocytes *ULG8* (PVP01_1452800), gametocyte development protein 1 *PvGDV1* (PVP01_0734100), high mobility group protein *B1* (PVP01_1302200), and inner membrane complex protein 1j *ALV7* (PVP01_1128100), as well as 11 male gametocyte genes including 6-cysteine protein *P47 and P48/45* (PVP01_1208000, PVP01_1208100), gamete egress sporozoite traversal protein *GEST* (PVP01_1258000), sexual stage antigen s16 (PVP01_0305600), male gamete fusion factor HAP2 (PVP01_0814300), and genes from the CPW-WPC family protein (PVP01_1215900, PVP01_1119500, and PVP01_1320100) were analyzed. To compare genetic diversity of the 28 target gametocyte genes, we combined each of the consensus sequences into single fasta file and used MAFFT v.7 default settings for sequence alignment. Both the nucleotide diversity (pi) and haplotype diversity (Hd) of these genes across all 131 samples were calculated using DnaSP (Rozas et al., 2017). The Pairwise-Deletion method was used for calculation and gaps were excluded in each pairwise comparison.

Phylogenetic Tree Reconstructions and Gene Network

To compare resolution power and genetic clustering pattern among the gametocyte genes, we constructed phylogenetic trees using Molecular Evolutionary Genetics Analysis (MEGA X) (Kumar et al., 2018). We selected the top five male and female gametocyte genes that revealed the highest haplotype and nucleotide diversity scores across all geographic locations. *Pvs*25 (PVP01_0616100) was used as a baseline for comparing phylogenetic resolution. For phylogenetic tree reconstructions, we first determined the best DNA substitution model for each of the genes. The maximum likelihood fits of 24 different nucleotide substitution models including the general time reversible model (TR), the Hasegawa-Kishino-Yano (HKY), the Tamura-Nei model (TN93), the Tamura 3-parameter model (T92), the Kimura 2-parameter model (K2), and the Jukes Cantor model (JC) was assessed by MEGA where the initial trees were automatically selected using the Neighbor-Join algorithms to a matrix of pairwise distances that were estimated using the Maximum Composite Likelihood approach. The best substitution model for each gene dataset was selected using a combination of the Bayesian Information Criterion (BIC), corrected Aikake Information Criterion (AICc), and log likelihood scores. Models with the lowest AICc were determined as the best substitution model for the gene. Using the optimal model, we constructed maximum likelihood phylogenetic trees in MEGA X. For the CPW-WPC family protein genes PVP01_1320100 and PVP01_1215900, the optimal model was the Kimura 2-Parameter model that is gamma distributed with invariant sites. For *PvULG8* (PVP01_1452800), we used the Hasegawa-Kishino-Yano substitution model that is gamma distributed with invariant sites. For *PvGDV1* (PVP01_0734100) and *Pvs230* (PVP01_0415800), we used the Tamura Nei substitution model that is gamma distribution with invariant sites. For each gene analysis, 100 bootstraps were performed to assess confidence of the genetic relationships among samples. We expanded on the resolving power in our phylogenetic trees by also constructing a gene transmission network using Strainhub (de Bernardi Schneider et al., 2020) for genes that showed clear genetic clustering among samples. Strainhub is a tool for creating gene flow networks using phylogenetic data and geographical metadata. The gene flow network was generated using the locations of our samples and calculating the source hub ratio (SHR) for each location. Numbers close to 1 represent places that are the sources of the transmissions, numbers close to 0.5 represent places that are the hubs through which transmissions pass and numbers that are close to 0 represent the sinks of the transmissions, places that are recipients.

Genetic Distance and Selection Analyses

To validate the phylogenetic trees, we assessed the degree of genetic differentiation among geographical isolates for the targeted genes by calculating an Analysis of Molecular Variance model (AMOVA) and pairwise fixation indices (F_{ST} statistic) between samples using Arlequin ver 3.5.2.2 (Excoffier and Lischer, 2010). A global locus by locus AMOVA, with 1000 permutations, was constructed using the gene sequence data from all 131 samples and variations within and between countries were estimated. In addition, a matrix of F_{ST} values, using 100 permutations and a significance level of 0.05, was measured to indicate the level of population divergence, with values ranging from 0 (no evidence of population divergence) to 1 (completely isolated). *P*-values in both the AMOVA and pairwise F_{ST} matrix were calculated to assess the level of significance. For each country, we also tested for positive selection among the gametocyte genes by the codon-based Z-test implemented in MEGA X. The Nei-Gojobori method and a bootstrap procedure of 100 replicates were conducted. To validate the results of Z-test, we further estimated the Tajima's D statistic using Tajima's D test and D, D*, F, and F* values using Fu and Li's tests implemented in DnaSP (Rozas et al., 2017).

Gametocyte Gene Expression Levels

To identify highly sensitive RNA biomarkers for male and female gametocytes, we examined the expression level of several male and female gametocyte genes and compared such with the standard *Pvs*25 in the a subset of 10 P. *vivax* samples from Ethiopia. A total of 10mL whole blood was preserved into sodium heparin from microscopy-confirmed *P. vivax* patients at hospitals in Jimma, Ethiopia, who had a minimum of 4,000 parasites/μL parasitemia and had not received prior antimalarial treatment. Scientific and ethical clearance was obtained from the institutional review boards of Jimma University, Ethiopia, and The University of North Carolina, Charlotte, USA. Written informed consent/assent for study participation was obtained from all consenting heads of households, parents/guardians (for minors under 18 years old), and from individuals who were willing to participate in the study. Upon collection, samples were cryo-preserved with 50% glycerolyte and stored in liquid nitrogen until *in vitro* culture. Prior to culture, samples were

thawed by adding 0.2V of 12% NaCl solution drop-by-drop followed by a 5-minute room temperature incubation. Ten-times volume of the 1.6% NaCl solution was then added drop-by-drop to the mixture and the samples were centrifuged at 1000 rcf for 10 minutes to isolate the red pellet. This process was repeated with 10x volume of the 0.9% NaCl. Following centrifugation, the supernatant was removed *via* aspiration, and 18mL of sterile complete IMDM per 1mL cryo mixture was added to each sample for a final hematocrit of 2%. 10% Giemsa thick microscopy slides were made to determine majority stage (**Supplementary File 1**) and duration of incubation, averaging 20-22 hours for majority trophozoites and 40-44 hours for majority ring. Samples were then incubated at 37°C in a 5% O_2, 5% CO_2 atmosphere to allow growth to the schizont stage.

In vitro maturation was validated through microscopic smears 17 hours after the initial starting time and subsequently checked every one to two hours. Cultured pellets were isolated *via* centrifugation and placed in 10x volume trizol for RNA extraction. RNA extraction was performed using Direct-zol RNA prep kit (Zymo Research) according to the manufacturer protocol, with two rounds of DNA digestion using the DNA-free kit (Zymo Research). Samples were analyzed with a nanodrop 2000 and RNA Qubit to ensure sample concentrations were above 150 ng total for library construction. For samples with no significant amount of DNA, RNA libraries were constructed using Illumina rRNA depletion library kits according to the manufacturer protocol. Sample reads were obtained using Illumina HiSeq 2x150bp configuration to obtain at least 35 million reads per sample. Sequence reads were aligned in HISAT2 to the P01 *P. vivax* reference genome and all human reads were filtered out using SAMtools. The alignment was mapped to the P01 reference annotation using the R package subread. Samples were then deconvoluted in CIBERSORTx based on *P. falciparum* homologs to obtain a transcription profile. Analyses of 25 targeted gametocyte genes including10 male and 15 female gametocyte genes were performed using DESeq2 to indicate expression levels among samples (see **Supplementary File 2** for accession numbers).

RESULTS

Comparison of Nucleotide and Haplotype Diversity

For the East African (Ethiopia, Sudan, Eritrea, and Uganda) *P. vivax* isolates, *Pvs*25 (PVP01_0616100) had a haplotype diversity (Hd) of 0.58 and nucleotide diversity (Pi) of 1.39×10^{-3} (**Figure 1**; **Supplementary File 3**). Of the 17 female gametocyte genes, *Pvs230* (PVP01_0415800; Hd: 0.999 and Pi: 1.24×10^{-3}), *GDV1* (PVP01_0734100; Hd: 0.965 and Pi: 2.86×10^{-3}), and *ULG8* (PVP01_1452800; Hd: 0.939 and Pi: 1.01×10^{-3}) were the most polymorphic compared to *Pvs*25 (**Figure 1**). Of the 11 male gametocyte genes, two CPW-WPC gamily protein genes PVP01_1215900 (Hd: 0.946 and Pi: 1.80×10^{-3}) and PVP01_1320100 (Hd: 0.940 and 9.58×10^{-4}) were most polymorphic (**Figure 1**). For the Southeast Asian *P. vivax*,

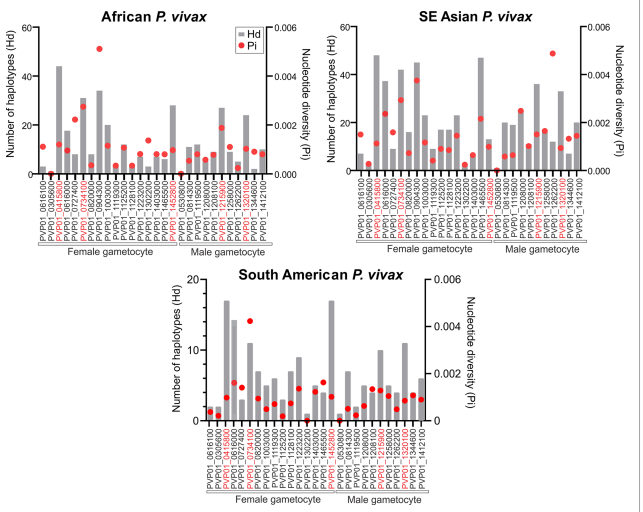

FIGURE 1 | Plots comparing the haplotype diversity and nucleotide diversity scores of all 17 female and 11 male gametocyte genes including *Pvs*25 (PVP01_0616100) among isolates obtained from East Africa, Asia, and South America. The gray bar represents haplotype diversity and the red dots represent the corresponding nucleotide diversity scores. Across all geographical isolates, *Pvs230* (PVP01_0415800), *GDV1* (PVP01_0734100), CPW-WPC family protein genes PVP01_1215900 and PVP01_1320100, *ULG8* (PVP01_1452800), and *Pvs*28 (PVP01_0616000) were highly polymorphic compared to the other gametocyte genes.

Pvs230 (PVP01_0415800; Hd: 0.998 and Pi:1.13×10^{-3}), CPW-WPC gamily protein gene PVP01_0904300 (Hd: 0.995 and Pi: 3.75×10^{-3}), and *ULG8* (PVP01_1452800; Hd: 0.998 and Pi: 2.16×10^{-3}) had the highest levels of polymorphism among the 17 female gametocyte genes. Of the 11 male gametocyte genes, PVP01_1215900 (Hd: 0.985 and Pi: 1.51×10^{-3}) and PVP01_1320100 (Hd: 0.963 and Pi: 9.36×10^{-4}) were most polymorphic. *Pvs*25 in the Cambodian and Thailand *P. vivax* was more polymorphic than the Ethiopian (Hd: 0.67 and Pi: 1.5×10^{-3}; **Figure 1**). and South American isolates (Hd: 0.247 and Pi: 3.74×10^{-4}; **Figure 1**). Similarly, for the South American (Peru and Panama) *P. vivax*, *Pvs230* (Hd: 0.96 and Pi: 9.83×10^{-4}) and *ULG8* PVP01_1452800 (Hd: 0.96 and Pi: 1.02×10^{-3}) were the most polymorphic among the 17 female gametocyte genes, consistent to the pattern observed in other geographic regions (**Figure 1**). Of the 11 male gametocyte genes, two CPW-WPC gamily protein genes PVP01_1215900 (Hd: 0.896 and Pi: 1.28×10^{-3}) and PVP01_1320100 (Hd: 0.896 and 8.54×10^{-4}) were the most polymorphic (**Figure 1**). Compared to *Pvs*25 and *Pvs*230, the other TBV gene candidate *Pvs*28 (PVP01_0616000) showed high polymorphisms among all geographic isolates, whereas *Pvs*48/45 (PVP01_1208100) was relatively conserved (**Figure 1**). Therefore, these six genes including *Pvs230* (PVP01_0415800), *GDV1* (PVP01_0734100), CPW-WPC family protein genes PVP01_1215900 and PVP01_1320100, *ULG8* (PVP01_1452800), and *Pvs*28 (PVP01_0616000) were selected for further analyses using *Pvs*25 (PVP01_0616100) as a standard.

Genetic Clustering Patterns and Resolving Power

For the three female (*Pvs230*, *GDV1*, and *ULG8*) and two male (PVP01_1215900 and PVP01_1320100 of the CPW-WPC gene family) gametocyte genes that showed high polymorphisms across the East African, Southeast Asian, and South American *P. vivax* isolates, the substitution model was first determined for each gene set prior to phylogenetic reconstructions. For genes PVP01_1215900 and PVP01_1320100, the Kimura two-parameter model with gamma distribution and invariant sites was selected as the best model based on the AICc and log likelihood scores (**Table 1**). For *Pvs25*, *GDV1*, and *Pvs*28, the Tamura 3 Parameter model was selected. For *ULG8*, the Hasegano-Kishino-Yano substitution model with gamma distribution and invariant sites was selected (**Table 1**). Most of the phylogenetic trees showed clear geographic differentiation between the Ethiopian and other geographical isolates (**Figure 2**), except for *GDV1* (PVP01_0734100) and CPW-WPC gene PVP01_1320100 where the Ethiopian *P. vivax* were mixed with the Southeast Asian and South American isolates (**Supplementary File 4**). In the *Pvs*25 (PVP01_0616100) phylogeny (**Figure 2**), four major clades were detected. The Ethiopian isolates were found in two of the clades, one of which contained also the Southeast Asian *P. vivax*. The South American samples were found exclusively in a separate clade. Within each clade, no resolution was detected among samples. In the *Pvs230* (PVP01_0415800) phylogeny (**Figure 2**), samples from Peru and Panama (South America) are clustered together sister to the Cambodian and Thailand (Southeast Asia) isolates. The Ethiopian *P. vivax* were genetically distant from these geographical isolates. Within each major clade, samples were well differentiated from one another. Likewise, in the *ULG8* (PVP01_1452800), *Pvs*28 (PVP01_0616000), and CPW-WPC gene PVP01_1215900 phylogenies (**Figure 2**), the Ethiopian *P. vivax* were clearly differentiated from the South American and Southeast Asian isolates. The South American and Southeast Asian isolates were well mixed with one another, though a small subclade containing only the Cambodian and Thailand samples was observed. For most genes, countries of the same continent were clustered together without clear differences. The resolving power of *Pvs230* (PVP01_0415800) and *ULG8* (PVP01_1452800) was the highest among all gametocyte genes (see **Supplementary File 5** for primer information of these two genes).

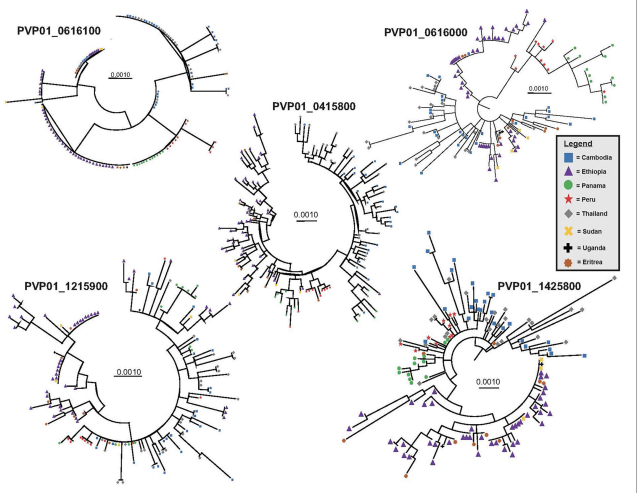

FIGURE 2 | Phylogenetic trees for four highly polymorphic gametocyte genes *Pvs230* (PVP01_0415800), *Pvs*28 (PVP01_0616000), *ULG8* (PVP01_1452800), and CPW-WPC family protein gene PVP01_1215900 illustrating the resolving power of gametocyte clones as compared to the standard *Pvs*25 (PVP01_0616100). Countries (Cambodia, Ethiopia, Eritrea, Panama, Peru, Thailand, Sudan, and Uganda) of the same continent were clustered together without clear differentiation. Among all, *Pvs*230 (PVP01_0415800) and *PvULG*8 (PVP01_1452800) showed most clear genetic differentiation between continents.

The AMOVA results showed that the variation observed between samples within each country was generally higher than that between countries (**Supplementary File 5**). Between countries, *Pvs25* (PVP01_0616100) and *Pvs28* (PVP01_0616000) have the greatest level of genetic differentiation (percent variation of 42% and 44%; $P<0.05$; **Supplementary File 5**), followed by *Pvs230* (PVP01_0415800) and *ULG8* (PVP01_1452800) with percent variations of about 32%. The pairwise F_{ST} values based on *Pvs230* (PVP01_0415800) and *ULG8* (PVP01_1452800) were on average 30%, consistent with their phylogenetic tree showing a much clearer distinction between countries than the other genes (**Figure 2**). Comparatively, *GDV1* (PVP01_1452800) and the CPW-WPC gene PVP01_1320100 had very low percentage variation between countries (percent variations of 15% and 18%, respectively) but much higher variations within each country (**Supplementary File 5**). Such low levels of genetic differentiation were consistent

TABLE 1 | Summary of the best DNA substitution model selected for each of the gametocyte genes and the metrics corrected AIC and log likelihood scores used in phylogenetic reconstructions.

Gene	Gene Description	Model Selection		
		Model	AICc	Log Likelihood
PVP01_0415800	6-cysteine protein P230, putative	Tamura Nei	34970.180	-17219.032
PVP01_0734100	Gametocyte development protein 1, putative GDV1	Tamura Nei	12213.975	-5840.786
PVP01_1452800	Upregulated in late gametocytes, putative ULG8	Hasegawa- Kishino-Yano	13204.928	-6337.308
PVP01_1215900	CPW-WPC family protein	Kimura 2 parameter	15092.227	-7283.982
PVP01_1320100	CPW-WPC family protein	Kimura 2 parameter	19448.261	-9462.261
PVP01_0616000	Ookinete surface protein P28, putative	Tamura 3 parameter	8054.830	-3764.160
PVP01_0616100	Ookinete surface protein P25	Tamura 3 parameter	5619.745	-2547.554

with their phylogenetic trees showing *P. vivax* from different countries in several clades without clear geographical distinction (**Supplementary File 3**). The gene transmission networks echoed the phylogenetic relationships. For instance, the network based on *Pvs230* (PVP01_0415800; **Figure 3A**) showed that Ethiopia and Thailand had a Source Hub Ratio (SHR) of 0.6 and 0.625, respectively, meaning that these two countries are likely the source of infections. Parasite gene flow was detected the highest among the East African countries, e.g., from Ethiopia to Sudan and Eritrea, as well as from Thailand to Cambodia in Southeast Asia (**Figure 3A**). Similar pattern was observed in the *PvULG8* (PVP01_1452800) based network (**Figure 3B**).

Signature of Positive Selection

Of the seven male and female gametocyte genes chosen, *ULG8* (PVP01_1452800) was detected with significant positive selection by the codon-based Z test among *P. vivax* isolates in Cambodia, Thailand, Ethiopia, and Eritrea ($P<0.01$; **Table 2**). Other genes including *GDV1* (PVP01_1452800), *Pvs230* (PVP01_0415800), and the two CPW-WPC genes PVP01_1215900 and PVP01_1320100 were not detected with positive selection (**Table 2**). The negative Tajima's D values detected in all the seven gametocyte genes indicated that these genes are not neutral.

Expression of Female and Male Gametocyte Genes

Eight out of ten *in vitro* samples from Ethiopia contained submicroscopic gametocytes based on *Pvs25* screening. Of these, two CPW-WPC genes PVP01_0904300 and PVP01_1119500 that represent the female and male gametocytes, respectively, were highly expressed (**Figure 4**)

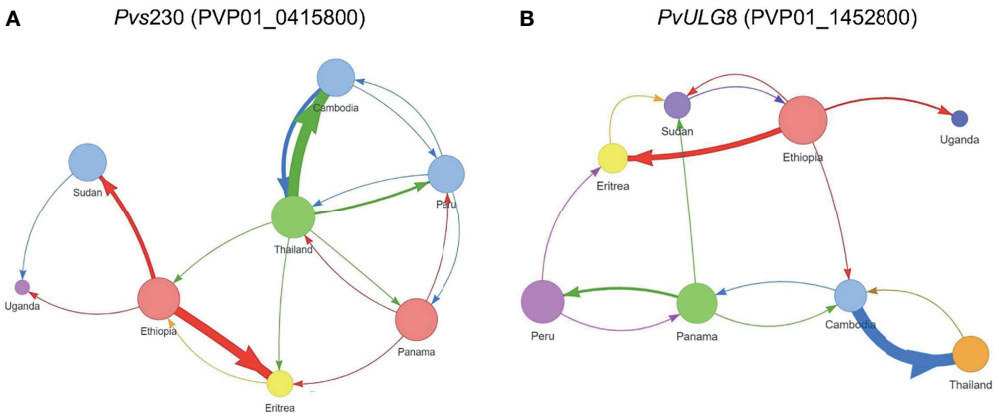

FIGURE 3 | Source Hub Ratio (SHR) transmission networks that further expand the resolving power for **(A)** *Pvs*230 (PVP01_0415800) and **(B)** *PvULG*8 (PVP01_1452800). The size of each node is proportional to the node's SHR value. Node colors were randomly assigned to each unique SHR value present in the plot to improve readability. Similarly, arrow colors are paired with the color of the node from which they begin to aid with interpretation. The weight of each arrow is proportional to the number of transitions between those two nodes. The position of nodes is arbitrary and is not equivalent to the position of sample sites in geographic space. More transitions were detected between African as well as between Asian countries, with few instances of gene flow from Asia to Africa. Ethiopia and Thailand had a Source Hub Ratio (SHR) of 0.6 and 0.625, respectively, meaning that these two countries are likely the source of infections. Parasite gene flow was detected the highest among the East African countries, e.g., from Ethiopia to Sudan and Eritrea, as well as from Thailand to Cambodia in Southeast Asia **(A)**. Similar pattern was observed in the *PvULG*8 (PVP01_1452800) based network **(B)**.

TABLE 2 | Positive selection test results based on codon-based Z test in MEGA X at 95% significance level.

Country	Positive selection *P*-value						
	Pvs230 PVP01_0415800	PvGDV1 PVP01_0734100	CPW-WPC protein gene PVP01_1215900	CPW-WPC protein gene PVP01_1320100	PvULG8 PVP01_1452800	Pvs25 PVP01_0616100	Pvs28 PVP01_0616000
Cambodia	0.482	1.000	1.000	1.000	0.001	1.000	0.128
Thailand	1.000	1.000	1.000	0.360	0.001	0.114	0.273
Peru	0.289	1.000	0.262	1.000	0.046	1.000	0.149
Ethiopia	0.440	1.000	0.363	0.239	0.002	0.051	0.314
Panama	0.027	1.000	1.000	1.000	0.227	1.000	0.179
Eritrea	0.369	1.000	1.000	1.000	0.001	0.042	1.000
Sudan	0.169	1.000	1.000	0.419	0.069	0.085	0.266
Uganda	0.356	1.000	0.229	0.038	0.042	1.000	1.000
Tajima's statistic	-1.78	-1.658	-1.687	-2.117	-1.740	-1.045	-1.319

Evidence for potential positive selection was observed in PvULG8 (PVP01_1452800) among the Cambodian, Thailand, and Ethiopian P. vivax isolates.

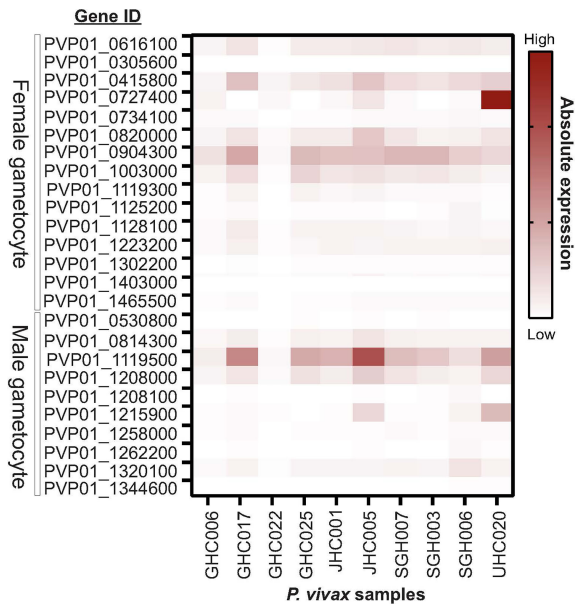

FIGURE 4 | Heatmap plot illustrating the expression level of 15 male and 10 female gametocyte genes for 10 Ethiopian *P. vivax in vitro* samples. Out of the 10 samples, eight contained gametocytes based on *Pvs25* screening whereas the other two samples (GHC006 and GHC022) did not express *Pvs25*. Amongst the 25 gametocyte genes, two CPW-WPC genes PVP01_0904300 and PVP01_1119500 that represent male and female gametocytes, respectively, were most highly expressed compared to the standard *Pvs25* across all eight gametocyte-positive samples.

and consistently higher than *Pvs*25 across all samples (**Figure 5**). Apart from PVP01_0904300, another female gametocyte gene *Pvs230* (PVP01_0415800) also showed a relatively high level of expression. *Pvs230* (PVP01_0415800) and PVP01_0904300 also revealed high levels of haplotype and nucleotide diversity (**Figure 1**) and high resolving power to *P. vivax* geographical samples (**Figure 2**).

DISCUSSION

The transmission of *P. vivax* relies on the development of gametocytes from committed schizonts in the human hosts and uptake by the *Anopheles* mosquitoes. Gametocytes especially in low-density and/or submicroscopic infections are hidden parasite reservoirs that can initiate transmission at any time. Thus, it is critical to utilize reliable biomarkers for their detection and effective malaria control intervention. *Pvs*25 and *Pvs*16 are two commonly used biomarkers for *P. vivax* gametocyte detection (Wampfler et al., 2013). However, they represent only female gametocytes and could underestimate the total gametocytes present in an infection. The proportions of infectious gametocytes as well as the ratio of male to female gametocytes can vary across infections, and in turn, determine parasitemia in the mosquito and the transmission potential (Tadesse et al., 2019). Gametocyte sex ratio positively correlates with gametocyte density with generally higher proportions of microgametocytes especially in low-density infections, which could result in optimal fertilization and higher gametocyte densities (Schall, 2000; Talman et al., 2004). Additionally, the ability to accurately detect male and female gametocytes will enable us to understand how anti-malarial drugs affect gametocyte production and transmission in natural infections (Henry et al., 2019) and develop transmission blocking therapies. In this study, we identify one male gametocyte CPW-WPC protein gene PVP01_1119500 and two female gametocyte genes *Pvs230* (PVP01_0415800) and CPW-WPC gene PVP01_0904300 that showed high expression relative to *Pvs*25. Both PVP01_0904300 and PVP01_1119500 belong to the CPW-WPC gene family, and *Pvs230* (PVP01_0415800) is a surface protein of *P. vivax* female gametocytes.

Better understanding of genetic polymorphisms in gametocyte genes have important implications on their potential in the development of transmission blocking vaccine candidates. *Pvs*25 is a highly expressed but conserved female gametocyte gene. It is one of the leading candidates for malaria TBV based on its high immunogenicity observed in animal model studies and high

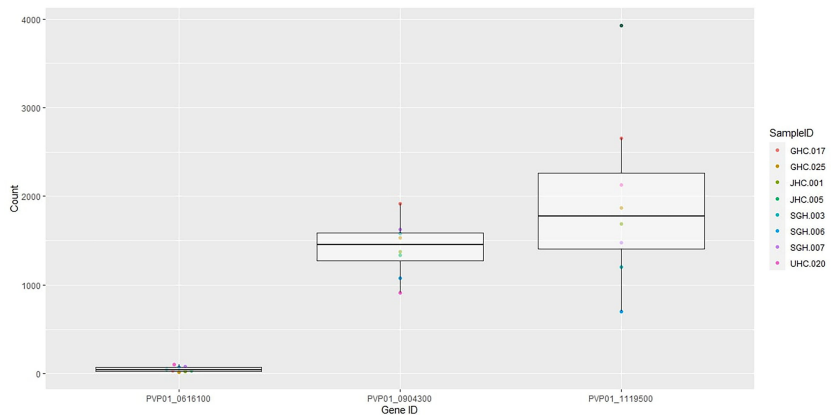

FIGURE 5 | Boxplots of *Pvs*25 (0616100) and the two CPW-WPC genes PVP01_0904300 and PVP01_1119500 showing gene expression level differences in the eight gametocyte-positive samples.

conservation among *P. vivax* isolates from endemic regions (Feng et al., 2011; Chaurio et al., 2016). In endemic regions of the Amazon in South America, *Pvs*25 was shown to be conserved among *P. vivax* isolates (Chaves et al., 2019). *Pvs*25 has been reported with positive selection in a previous study (Chaurio et al., 2016). The present study indicated that *Pvs*25 is less polymorphic than other female and male gametocyte genes and is not neutral. *P. vivax* isolates from the same country were more closely related or genetically similar than from different countries. These findings suggested that selection may have contributed to the conserved nature of this gene.

*Pvs*48/45, a male gamete fertilization factor, is another main transmission blocking vaccine candidate (Feng et al., 2015). Our analyses indicated a relatively conserved nature of this gene among global isolates, agrees with a previous study that indicated low levels of genetic diversity in *Pvs48/45* among 200 P. *vivax* isolates from temperate and subtropical populations in China (Feng et al., 2015). Of the 14 SNPs, 11 were nonsynonymous with an overall nucleotide diversity of 0.0012 across the isolates. At the continental level, F_{ST} values based on *Pvs*48/45 widely ranged from 0.34 to 0.90 among samples from several countries in Asia, America, and Oceania, with an overall F_{ST} of 0.665 (Feng et al., 2015). Though earlier studies of isolates from China showed *Pvs*48/45 undergoing potential positive selection with the distribution of polymorphic sites concentrated in domain II of the gene (Feng et al., 2015), the present study did not detect significant positive or non-neutral selection in this gene. High levels of antibodies against Pfs48/45 and Pfs47 were previously reported in *P. vivax* endemic regions (Anthony et al., 2007). Immuno-epidemiological studies of the parasite sexual stage antigens including Pvs48/45 and Pvs230 showed that antibodies against these antigens are present in people living in *P. vivax* endemic areas and associated with transmission blocking activities (Bousema et al., 2006; Drakeley CJ et al., 2006; Bousema et al., 2010; Feng et al., 2015; Jones et al., 2015). It is possible that these gene antigens illicit host antibody responses and impose selection, leading to scarce genetic polymorphisms in the parasite populations.

Gametocyte production is critical for sexual reproduction of the parasite and subsequent transmission to a new host. It is essential to identify gametocyte clones and the origin of transmission to effectively identify transmission reservoirs in both symptomatic and asymptomatic infections. Prior studies have demonstrated that low density *P. vivax* gametocytes in asymptomatic carriers significantly contribute to transmission and genetic diversity (Bousema et al., 2014; Abdelraheem et al., 2018). Lower genetic diversity in *P. vivax* populations is correlated with lower transmission frequencies and low amounts of imported parasites, resulting in fewer clones (Nguitragool et al., 2017; Kepple et al., 2021). Our findings showed that two female gametocyte genes *Pvs*230 (PVP01_0415800) and *ULG*8 (PVP01_1425800) were highly polymorphic compared to *Pvs25*, which are potential DNA markers for determining the source of *P. vivax* gametocytes and differentiating gametocyte clones within and between hosts. Previous studies indicated limited polymorphism in *Pvs*230, making this gene a potential candidate of malaria transmission blocking vaccine (Doi et al., 2011). In a study involving 112 full length *Pvs*230 sequences of *P. vivax* isolates from regions in South America, Southeast Asia, Indo-West Pacific, and Madagascar, polymorphism of this gene was much lower than the bloodstage antigen genes (Doi et al., 2011), likely due to the fact that bloodstage antigens are targets of host antibody responses and high genetic diversity in the antigen genes would facilitate host immune response evasion (Doi et al., 2011). Our findings did not support the conservative nature of *Pvs*230 among different geographical isolates. Instead, we detected high genetic differentiation of *Pvs230* among *P. vivax* from the same geographic region. Our transmission network based on *Pvs230* further showed as high as 13 transitions between Cambodia and Thailand, a high level of gene flow within Southeast Asia. On the other hand, the limited gene flow among the continental isolates may explain the differentiation of *Pvs230*. No significant positive selection was detected in *Pvs230* in this study, likely because this protein located on the surface of gametocytes is involved in gamete recognition and fertilization and could be highly divergent among parasite populations (Tsaur et al., 2001; Palumbi, 2009; Doi et al., 2011). Nevertheless, the high polymorphisms observed in *Pvs230* makes this gene a less viable candidate for TBV.

The CPW-WPC proteins were previously shown to be expressed on the surface of *P. falciparum* and *P. berghei* ookinetes and may play a role in transmission by the mosquito (Kangwanrangsan et al., 2013; Rao et al., 2016). In Thailand, CPW-WPC genes were detected with significant positive selection likely due to their role in mosquito transmission (Diez Benavente et al., 2017), which may raise concerns on the reliability of using CPW-WPC protein genes as biomarkers. *PvULG*8 (PVP01_1452800) was detected with positive selection in the Cambodian, Thailand, and Ethiopian *P. vivax*. The ortholog *PfULG*8 is a member of the *P. falciparum* gene family encoding the CPW-WPC proteins. An integrated transcriptomic and proteomic analysis of *P. falciparum* gene expression showed that transcripts from nine CPW-WPC genes predominantly accumulate in female gametocytes and are subjected to translational repression (Lasonder et al., 2016; Siciliano et al., 2017). Though these transcripts are produced in gametocytes, they are translated only in the mosquito parasite stages (Siciliano et al., 2017). Further studies examining diversity of CPW-WPC genes, especially PVP01_0904300 and PVP01_1119500, are needed to understand the full potential of these two genes for gametocyte detection. In this study, the transcriptomic data were based on a small number of Ethiopian isolates. In the future, it is important to correspond SNPs obtained from gDNA with SNPs on expressed transcripts in gametocytes and other epidemiological features such as the infectiousness and host immune responses of broader samples in order to understand better the functional significance of polymorphisms detected at the DNA level.

CONCLUSION

Conventional methods of detecting *P. vivax* gametocytes may underestimate the level of transmission reservoirs. A new approach is needed to better detect and monitor the transmission

potential especially in countries approaching malaria elimination. The present study examined polymorphisms and resolving power of 28 male and female gametocyte genes through phylogenetic and selection testing. Given high genetic differentiation and clear clustering of gametocyte clones by *Pvs230* (PVP01_0415800) and *PvULG8* (PVP01_1452800), these two genes could provide a better detection method of identifying transmission reservoirs and source of transmission for symptomatic and asymptomatic malarial infections. Transcriptomic data of a few Ethiopian *P. vivax* samples showed a high expression of *Pvs230* (PVP01_0415800) as compared to *Pvs*25, suggesting its potential as a sensitive RNA marker for detecting low-gametocytemia infections. It is noted that *PvULG8* was detected with positive selection in the Southeast Asian and African *P. vivax*, and the underlying reason warrants further investigation. Future studies on gametocyte reservoirs especially in asymptomatic and/or low-density infections using *Pvs230* and *PvULG8* may shed light on the importance of these genes as biomarkers in the detection of gametocytes.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/**Supplementary Material**.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Scientific and ethical clearance was obtained from the institutional review boards of Jimma University, Ethiopia, and The University of North Carolina, Charlotte, USA. Written informed consent/assent for study participation was obtained from all consenting heads of households, parents/guardians (for minors under 18 years old), and from individuals who were willing to participate in the study. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

Conceptualization, LG, DY, and EL. Resources, DJ, DY, and EL. Conduct experiment and data analyses, AF, DK, JW, GK, CF, and AA. Writing—original draft preparation, AF, DK, JW, GK, and EL. Writing—review and editing, DJ, LG, DY, and EL. Funding acquisition, EL. All authors have read and agreed to the published version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2021.789417/full#supplementary-material

Supplementary File 1 | Giemsa-stained smears for *in vitro* maturation of *P. vivax* samples.

Supplementary File 2 | Accession numbers for the Ethiopia *P. vivax* transcriptomic data used in gene expression plots.

Supplementary File 3 | Nucleotide and haplotype diversity scores of 28 gametocyte genes in the East African, Southeast Asian, and South American *P. vivax* isolates by DNASP analyses.

Supplementary File 4 | Phylogenetic trees for two gametocyte genes *GDV1* (PVP01_0734100) and CPW-WPC gene PVP01_1320100 that showed the mix of Ethiopian *P. vivax* together with the Southeast Asian and South American isolates in major clades.

Supplementary File 5 | Primer information of two gametocyte genes *Pvs230* (PVP01_0415800) and *PvULG8* (PVP01_1452800).

Supplementary File 6 | Pairwise F_{ST} matrix and results of AMOVA between all countries of seven targeted gametocyte genes. *P*-values were indicated to show the level of significance.

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Revisiting the Effect of Pharmaceuticals on Transmission Stage Formation in the Malaria Parasite *Plasmodium falciparum*

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Malaria parasites rely on specialized stages, called gametocytes, to ensure human-to-human transmission. The formation of these sexual precursor cells is initiated by commitment of blood stage parasites to the sexual differentiation pathway. *Plasmodium falciparum*, the most virulent of six parasite species infecting humans, employs nutrient sensing to control the rate at which sexual commitment is initiated, and the presence of stress-inducing factors, including antimalarial drugs, has been linked to increased gametocyte production *in vitro* and *in vivo*. These observations suggest that therapeutic interventions may promote gametocytogenesis and malaria transmission. Here, we engineered a *P. falciparum* reporter line to quantify sexual commitment rates after exposure to antimalarials and other pharmaceuticals commonly prescribed in malaria-endemic regions. Our data reveal that some of the tested drugs indeed have the capacity to elevate sexual commitment rates *in vitro*. Importantly, however, these effects are only observed at drug concentrations that inhibit parasite survival and only rarely result in a net increase of gametocyte production. Using a drug-resistant parasite reporter line, we further show that the gametocytogenesis-promoting effect of drugs is linked to general stress responses rather than to compound-specific activities. Altogether, we did not observe evidence for mechanistic links between the regulation of sexual commitment and the activity of commonly used pharmaceuticals *in vitro*. Our data hence does not support scenarios in which currently applied therapeutic interventions would promote the spread of drug-resistant parasites or malaria transmission in general.

Keywords: sexual commitment, *Plasmodium falciparum*, gametocytes, malaria transmission stages, malaria, antimalarials, high content imaging (HCI)

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INTRODUCTION

Causing an estimated 200 million clinical cases and more than 400`000 deaths annually, malaria represents one of the major threats to global public health (WHO, 2020). Malaria parasites resistant to current drug interventions, including the frontline artemisinin-based combination therapies (ACTs), are emerging and endanger malaria eradication campaigns (Ippolito et al., 2017). Among the six *Plasmodium* species infecting humans, *P. falciparum* is the most virulent and accounts for the majority of severe and lethal malaria cases (WHO, 2020). Following the injection of sporozoites by an infected *Anopheles* mosquito, parasites reproduce within hepatocytes before initiating the symptomatic phase of infection in the human blood. The latter is characterized by continuous rounds of erythrocyte invasion, asexual replication, host cell rupture, and the release of merozoites ready to invade new red blood cells (RBCs). During each of these 48-hour long intra-erythrocytic replication cycles, a small subset of parasites switches away from asexual replication and instead commits to sexual development, resulting in the formation of transmissible gametocytes (Josling et al., 2018). *P. falciparum* gametocytes sequester in deep tissue, including the bone marrow parenchyma, where they undergo a series of developmental steps (I-V) before re-entering the blood stream as mature and transmission-competent stage V gametocytes after 10-12 days (Hawking et al., 1971; Ngotho et al., 2019). While gametocytes represent the only cell type that is infectious to mosquitoes, they are non-replicative. Investments into gametocytogenesis thus come at the expense of reduced vegetative growth and *P. falciparum* employs sophisticated mechanisms to regulate this trade-off between within-host replication and between-host transmission (Carter et al., 2013).

Sexual commitment requires expression of AP2-G – a member of the ApiAP2 family of DNA-binding factors (Kafsack et al., 2014; Sinha et al., 2014). This master regulator of sexual commitment primes asexually replicating parasites to produce sexually committed ring stage progeny that exit the cell cycle and undergo gametocyte development (Josling et al., 2018). In addition to this mechanism referred to as `next cycle conversion`, the immediate induction of gametocytogenesis in ring stages has also been observed, albeit this `same cycle conversion` is induced at a low rate and has so far only been described *in vitro* (Bancells et al., 2019). In the predominant next cycle conversion route, the decision of whether to stay within the asexual pathway or commit to the production of gametocytes is made in early schizonts at 36 +/- 4 hours post-erythrocyte invasion (hpi) (Brancucci et al., 2017). While AP2-G expression is initiated almost simultaneously, gametocyte differentiation will only start after completion of the current intra-erythrocytic developmental cycle (IDC) and the invasion of sexually committed merozoites into new RBCs (Ngotho et al., 2019). On the molecular level, the process of sexual commitment is under epigenetic control. It involves the activity of a number of well-characterized factors, including heterochromatin protein 1 (HP1), histone deacetylase 2 (HDA2) and gametocyte development protein 1 (GDV1) (Josling et al., 2018). These factors act in concert to control transcriptional activity at the *ap2-g* locus. The *ap2-g* locus is generally kept in a silenced state marked by the presence of histone 3 tri-methylated at lysine 9 (H3K9me3) and the histone code eraser and reader proteins HDA2 and HP1, respectively (Flueck et al., 2009; Lopez-Rubio et al., 2009; Salcedo-Amaya et al., 2009; Brancucci et al., 2014; Coleman et al., 2014). In contrast, GDV1 counteracts the silencing of *ap2-g* by evicting HP1 from the *ap2-g* locus, thus triggering AP2-G expression and sexual commitment in schizonts (Eksi et al., 2012; Filarsky et al., 2018; Usui et al., 2019). Through an auto-regulatory positive feedback loop, AP2-G expression increases further and peaks in the sexually committed ring stage parasites where it binds to and regulates the expression of genes linked to gametocytogenesis (Bancells et al., 2019; Josling et al., 2020; Llorà-Batlle et al., 2020). Together, these studies revealed that sophisticated epigenetic mechanisms are in place to balance asexual reproduction versus investments into transmission stage formation (Carter et al., 2013; Josling et al., 2018).

P. falciparum parasites invest surprisingly little into transmission. In fact, sexual commitment rates (SCRs) in parasite populations typically remain at a low single-digit percentage *in vitro* and *in vivo* (Taylor and Read, 1997; Buchholz et al., 2011; Carter et al., 2013; Brancucci et al., 2015; Usui et al., 2019). This reproductive restraint can be lifted under certain circumstances, resulting in greatly enhanced sexual commitment of parasites and conversion rates above 30% under specific *in vitro* conditions (Neveu et al., 2020). These include, but are not restricted to, high parasite densities (Bruce et al., 1990; Delves et al., 2016), exposure to *P. falciparum*-conditioned (nutrient-depleted) medium (Williams, 1999; Dyer and Day, 2003; Fivelman et al., 2007; Brancucci et al., 2015), endoplasmic reticulum stress (Chaubey et al., 2014) or the uptake of extracellular vesicles derived from infected RBCs (iRBCs) (Mantel et al., 2013; Regev-Rudzki et al., 2013). These observations contributed to the appreciation that intra-erythrocytic parasites are able to interact with and respond to their environment. Indeed, recent studies revealed that blood stage parasites modulate specific transcriptional programs in response to nutrient availability and other environmental cues (Kumar et al., 2021). Importantly, *P. falciparum* was found to metabolize the host-derived serum lipid lysophosphatidylcholine (lysoPC) in the Kennedy pathway and to induce sexual commitment under conditions that limit activity of this metabolic route (Brancucci et al., 2017). Expression of GDV1, the earliest known marker of sexual commitment, is induced at low lysoPC concentrations, implying a direct link between the epigenetic mechanisms that control gametocyte production and parasite nutrient-sensing (Neveu et al., 2020).

In addition, several lines of evidence suggest that some antimalarial drugs may interfere with the process of sexual commitment (Buckling et al., 1997; Puta and Manyando, 1997; Buckling et al., 1999a; Peatey et al., 2009; Baker, 2010; Portugaliza et al., 2020). Increased gametocyte production was observed *in vitro* upon treatment with artemisinin, mefloquine, chloroquine, primaquine, atovaquone and piperaquine (Peatey et al., 2009).

Furthermore, treatment with sub-curative doses of the widely used drugs chloroquine (Buckling et al., 1997) and sulfadoxine/pyrimethamine ("Fansidar") (Puta and Manyando, 1997) have been associated with increased gametocyte production *in vivo*. Using a transgenic reporter cell line for the quantification of sexual conversion rates (Portugaliza et al., 2019), the Cortés laboratory recently confirmed a gametocytogenesis-inducing effect for the frontline antimalarial dihydroartemisinin (DHA) (Portugaliza et al., 2020). In this study, short pulses of sub-curative DHA concentrations applied to trophozoites induced sexual commitment *in vitro*, but this effect was not observed when ring or schizont stage parasites were exposed to the drug. Together, these studies raise legitimate concerns about whether therapeutic interventions may promote gametocytogenesis and hence malaria transmission. However, the mechanisms underlying such potential drug-induced increases in gametocyte production are hitherto unknown. On the one hand, antimalarials may promote sexual commitment by inducing general cellular stress responses. On the other hand, it is conceivable that gametocytogenesis may be induced by drug-specific modulation of the molecular process underlying sexual commitment – i.e. independent of the toxic effect of the drug. In this latter scenario, drug-resistant parasites would be expected to shift their investment towards gametocyte formation under drug pressure, which may eventually promote transmission and spread of drug resistance (White, 2008). Indeed, strains carrying specific drug resistance mutations have been associated with increased mosquito transmission following antimalarial treatment (Bell et al., 2012). Furthermore, chloroquine-resistant parasites showed increased gametocytaemia and mosquito infectivity following drug treatment compared to chloroquine-sensitive strains (Hogh et al., 1998). Other efforts, however, failed to confirm the specific induction of gametocyte production in drug-resistant parasites after exposure to sub-curative chloroquine and pyrimethamine concentrations *in vitro* (Reece et al., 2010). Hence, while drug-resistant parasite strains may have an increased transmission potential, it remains unclear whether this would be linked to higher parasite survival rates under drug pressure or truly increased rates of commitment to gametocyte formation.

The SCR reflects the proportion of schizonts within a given IDC that commit to gametocytogenesis and produce sexual ring stage progeny. Accurate calculation of SCRs hence relies on the simultaneous quantification of either asexually and sexually committed schizonts in the commitment cycle or of asexual parasites and early stage gametocytes in the immediate progeny, i.e. within the first 48 hours after invasion. However, since gametocytes are morphologically indistinguishable from asexual parasites until day three of sexual differentiation (stage II), the reliable determination of SCRs has traditionally been very laborious and technically challenging. Several laboratories have therefore developed flow cytometry-based assays that allow for an accurate measurement of gametocytaemia in parasites expressing fluorophores or fluorophore-tagged gametocyte markers under the control of ectopic gametocyte-specific promoters (Dixon et al., 2009; Peatey et al., 2009; Buchholz et al., 2011; Brancucci et al., 2015; Portugaliza et al., 2019). These assays enable distinguishing gametocytes from asexual stage parasites before they become morphologically distinct and therefore allow minimizing effects that may confound a precise determination of SCRs. Potential confounding factors include (i) the erroneous counting of gametocytes originating from multiple previous IDCs, (ii) the effect of multiplying asexual parasites and, in case of probing drugs or drug-like molecules, (iii) the potential lack of activity of compounds on early gametocyte survival. Because of the reduced sensitivity of gametocytes to most antimalarials (Peatey et al., 2012; Plouffe et al., 2016), it is important to identify and exclude confounding effects emerging from differential survival between asexual and sexual stage parasites. Hence, compared to standard light microscopy-based setups, flow cytometry-based assays greatly improved the accuracy and likewise the throughput of measuring SCRs in parasite populations (Dixon et al., 2009; Peatey et al., 2009; Buchholz et al., 2011; Brancucci et al., 2015; Portugaliza et al., 2019).

Here, we developed a novel high content imaging (HCI) assay for the precise quantification of sexual commitment rates in *P. falciparum* parasites. This assay identifies sexually committed ring stage parasites based on the expression of endogenous mScarlet-tagged AP2-G, the earliest and most specific marker for sexually committed ring stages (Kafsack et al., 2014; Sinha et al., 2014; Bancells et al., 2019; Llorà-Batlle et al., 2020). Given the potential impact of antimalarials on malaria transmission, we used this assay to test a comprehensive panel of drugs for their possible effects on sexual commitment. In addition, we also included a diverse collection of other therapeutics that are commonly prescribed in malaria-endemic regions, including antihelminthics and analgesics, to account for a possible role of general stress-inducing factors on the sexual commitment process. Our results provide a systematic evaluation of the links between drug treatment and sexual commitment and suggest that antimalarial drug treatment does not promote transmission stage formation in *P. falciparum*.

MATERIALS AND METHODS

Parasite Culture

Intra-erythrocytic *P. falciparum* stages were cultured and synchronized as described (Jensen and Trager, 1978; Lambros and Vanderberg, 1979). Generally, parasites were grown in AB+ or B+ human RBCs (Blood Donation Center, Zürich, Switzerland) at a hematocrit of 5% in parasite culture medium consisting of 10.44 g/L RPMI-1640, 25 mM HEPES, 100 µM hypoxanthine, 24 mM sodium bicarbonate, 0.5% AlbuMAX II (Gibco #11021-037) and 0.1 g/L neomycin. The medium was further complemented with 2 mM choline chloride (Sigma #C7527) to maintain low background sexual commitment rates as observed in the presence of human serum (Brancucci et al., 2017). Cultures were gassed with 3% O_2, 4% CO_2 and 93% N_2 and incubated in an airtight incubation chamber at 37°C.

Cloning of Transfection Constructs

CRISPR/Cas9-based genome engineering of the NF54/ap2g-mScarlet, TM90C2B/ap2g-mScarlet and NF54/ap2g-re9h parasites was performed using a two-plasmid approach as previously described (Brancucci et al., 2017; Filarsky et al., 2018). This system is based on co-transfection of a suicide and a donor plasmid. The suicide plasmid contains the expression cassettes for the Cas9 enzyme, the single guide RNA (sgRNA) and the human dihydrofolate reductase (hDHFR) resistance marker (pH-gC). A pD-derived donor plasmid was used for homology-directed repair of the Cas9-induced DNA double strand break (Filarsky et al., 2018).

The pH-gC_ap2g-3' suicide plasmid targeting the 3' end of *pfap2-g* has been described previously (Brancucci et al., 2017). The donor plasmid pD_ap2g-mScarlet was generated by assembling (i) the BamHI and SfoI-digested pD_ap2g-gfp plasmid (Brancucci et al., 2017) with a PCR product containing (ii) the *mScarlet* sequence preceded by nucleotides encoding a GSAG linker using the primers mScarlet-F and mScarlet-R amplified from a *P. falciparum* codon-optimized synthetic *mScarlet* sequence (Boltryk et al., 2021), and (iii) the 3' homology region amplified from the pD_ap2g-gfp plasmid (Brancucci et al., 2017) using the primers ap2-g-3'-F and ap2-g-3'-SfoI-R in a Gibson reaction (Gibson et al., 2009). The donor plasmid pD_ap2g-re9h was generated by assembling two PCR products using the primers iso_re9h-F and iso_re9h-R amplified from pD_ap2g-mScarlet, and (Gutman et al., 2009) re9h-F and re9h-R to amplify the *re9h* fragment (Branchini et al., 2010) from pTRIX2-re9h (Lewis et al., 2014). The sequence of the self-cleaving peptide T2A was included in the primers iso_re9h-R and re9h-F (Liu et al., 2017). Primer sequences used for cloning are listed in **Table S1**.

Transfection and Selection of Gene Edited Parasites

P. falciparum transfection using the CRISPR/Cas9 suicide and donor plasmid approach was performed as described previously (Filarsky et al., 2018). Briefly, 50 µg of each of the suicide plasmid (pH-gC_ap2g-3') and the respective donor plasmid (pD_ap2g-mScarlet or pD_ap2g-re9h) were co-transfected. Transgenic parasites were selected with 4 nM WR 99210 24 h after transfection for 6 days. Transgenic populations were usually obtained 2–3 weeks after transfection and correct editing of the *ap2-g* locus was then confirmed by PCR on gDNA. Primer sequences used for these PCRs are listed in **Table S1**.

Drug Assays

The screening for sexual commitment-inducing compounds was performed in a 96-well plate format and compounds were tested in twelve concentrations using two-step or three-step serial dilutions. One well per condition was measured for each independent biological replicate. Stock solutions of 10 mM drug were prepared in DMSO, except for chloroquine, acetaminophen, Aspirin, diclofenac, ibuprofen and piparaquine (prepared in -SerM). Working solutions were prepared in -SerM medium immediately before the experiment and 100 µL each was dispensed into the wells of a cell culture plate (Corning Incorporated, 96-well cell culture plate, flat bottom, REF 3596). Synchronous asexual parasites at 20-26 hpi and a parasitaemia of 0.5-1% were washed and resuspended in -SerM medium complemented with 4 mM choline chloride at 2.5% hematocrit. 100 µL parasite suspension was then added to each well and gently mixed with the compounds. As a positive control for sexual commitment-inducing conditions, parasites resuspended in -SerM medium lacking choline chloride were used. Plates were gassed and incubated in an airtight incubation chamber at 37°C for 48 hours.

Quantification of Parasite Survival

At 20-26 h after reinvasion into new RBCs (i.e. 48 hours after the start of the assay), the parasitaemia was determined using flow cytometry. To this end, 40 µl parasite suspension was transferred from each well to a new 96-well plate (Corning Incorporated, 96-well cell culture plate, round bottom, REF 3788) and the samples were stained for 20 min with 40 µl 2X SYBR Green DNA stain (Invitrogen S7563) and then washed twice in 200 µl PBS. Plates were spun at 280 g for 2 min in between each step. To determine the parasitaemia, 200`000 events per sample were measured using the MACS Quant Analyzer 10. Data was analyzed using the FlowJo_v10.6.1 software. The gating strategy removed small debris and doublets (two cells per measurement), and iRBCs were distinguished from uninfected RBCs based on the SYBR Green intensity (**Figure S6**). Mean survival rates were calculated relative to -SerM/choline controls

$$\frac{parasitemia\ (test\ condition)}{parasitemia\ (-SerM/choline)} * 100$$

from three independent biological replicates. Curve fitting was performed using non-linear, four parameter regression model with variable slope (Graph Pad Prism, version 8.2.1).

Quantification of Sexual Commitment Rates by High Content Imaging

The SCR is defined as the proportion of sexually committed parasites in the total population. High content imaging (HCI) microscopy and automated image analysis were used to detect the number of all parasites based on DNA staining alone, whereas the sexually committed parasites were recognized *via* both DNA staining and AP2-G-mScarlet fluorescence. At 20-26 hpi after reinvasion into new RBCs (i.e. 48 hours after the start of the assay), cultures were stained with Hoechst (2.5 µg/mL) for 20 min and washed twice in 200 µL PBS. Plates were spun at 280 g for 2 min in between each step. The cultures were then diluted in PBS to a hematocrit of 0.075% and 200 µL were transferred to a clear-bottom 96-well HCI plate (Greiner CELLCOAT microplate 655948, Poly-D-Lysine, flat µClear bottom). Cells were allowed to settle for 15 min before image acquisition with an ImageXpress Micro widefield high content screening system (Molecular Devices) in combination with the MetaXpress software (version 6.5.4.532, Molecular Devices) and a Sola SE solid state white light engine (Lumencor). Filtersets for Hoechst (Ex: 377/50 nm, Em: 447/60 nm) and mScarlet (Ex: 543/22 nm, Em: 593/40 nm) were used with exposure times of 80 ms and 600 ms, respectively. 36 sites per well were imaged using a Plan-Apochromat 40x objective (Molecular

Devices, cat# 1-6300-0297). Automated image analysis was performed using the MetaXpress software (version 6.5.4.532, Molecular Devices). Hoechst-positive as well as mScarlet-positive parasites were identified using a modular image analysis workflow built within the ImageXpress software (Molecular Devices) described in **Table S2**, allowing for the calculation of sexual commitment rates (i.e. the proportion of Hoechst/mScarlet double-positive cells among all Hoechst-positive cells). SCRs were calculated as follows

$$\frac{mScarlet-positive\ cells(test\ condition)}{Hoechst-positive\ cells(test\ condition)} * 100$$

Mean relative SCRs were calculated relative to -SerM controls

$$\frac{SCR(test\ condition)}{SCR(-SerM)} * 100$$

from three independent biological replicates.

Quantification of RE9H Luminescence Intensity

At 20-26 h after reinvasion into new RBCs (i.e. 48 hours after the start of the assay), 180 µL of NF54/ap2g-re9h parasite culture was transferred from each well to a black 96-well plate (Greiner CELLSTAR microplate 655086, F-bottom, black) and incubated with 20 µL D-Luciferin (3.75 mg/mL in PBS) (Perkin Elmer, catalog# 122799) for 5-10 min at room temperature. Luminescence intensities were then measured using an intra-vital imaging system (Perkin Elmer, Lumina II) by exposing the plates for 3 min. Luminescence counts per well were determined by fitting a grid over the plate using the software Living Image (version 4.7.2).

Quantification of Gametocyte Production Rates by Light Microscopy

At 20-26 hpi, parasites were exposed to either -SerM or -SerM/choline conditions, gassed and incubated in an airtight incubation chamber at 37°C for 48 hours to complete the IDC and produce ring stage progeny. At this time point, parasitaemia was determined using Giemsa-stained blood smears (day 1). The culture medium was replaced with culture medium containing 2 mM choline and 50 mM N-acetyl-D-glucosamine to eliminate asexual parasites and the cultures were incubated for 72 hours with daily medium changes (Fivelman et al., 2007). Gametocytaemia was determined by Giemsa-stained blood smears on day 4 (stage II gametocytes). Sexual commitment rates were calculated by dividing the gametocytaemia determined on day 4 by the total parasitaemia determined on day 1 using results obtained from three independent biological replicates.

RESULTS

An Assay to Quantify Sexual Commitment Rates in *P. falciparum*

De-repression of the *ap2-g* locus marks the earliest known transcriptional event of sexual commitment. Here, we used a CRISPR/Cas9 gene editing strategy to fuse the *ap2-g* gene in frame to a sequence coding for the red fluorescent protein mScarlet (**Figure 1A** and **Supplementary Figures 1A, B**). Using these NF54/ap2g-mScarlet parasites, we established an HCI-based assay to quantify the SCRs in live parasite populations *via* monitoring expression of the fluorescently tagged AP2-G-mScarlet reporter protein (**Supplementary Figure 1C**). In brief, the sexual commitment assay is initiated by seeding highly synchronous NF54/ap2g-mScarlet parasites in 96-well flat-bottom cell culture plates at the late ring/early trophozoite stage (20-26 hpi) at 1.25% haematocrit and 0.5-1% parasitaemia and exposing them to test conditions. After a 48-hour incubation period, i.e. during early intra-erythrocytic development of the subsequent generation, parasites are stained using the DNA dye Hoechst and transferred to a 96-well imaging plate at a haematocrit of 0.075%. The proportion of sexually committed ring stages/early stage I gametocytes (AP2-G-mScarlet-positive) among all iRBCs (Hoechst-positive) is quantified by fluorescence HCI microscopy. Imaging of 36 sites per well allows capturing information for 5000-10`000 iRBCs. (**Figure 1B**). At the same time point, parasitaemia is determined for each well of the cell culture plate in parallel by flow cytometry of SYBR Green-stained iRBCs to assess potential effects of the test conditions on parasite survival (multiplication).

To establish and validate this HCI assay, we used parasites cultured in serum-free medium (-SerM) either in presence or absence of choline, the commitment-inhibiting metabolite of lysoPC (Brancucci et al., 2017). As expected, these +/- choline control conditions (-SerM/choline; -SerM) resulted in consistently low and high SCRs, respectively (**Figure 1C**), and revealed high assay robustness throughout the experiments presented here (Z'-factor of 0.79 (SD: 0.09), **Figure 1D**).

We used serum-free medium for two main reasons: First, the -SerM and -SerM/choline conditions allow for a controlled experimental setup across biological replicate experiments and, compared to serum-complemented medium, are independent of confounding factors such as varying levels of lysoPC and choline in serum deriving from different donors. Second, the balance between cytotoxic effects of lysoPC and activity on *P. falciparum* sexual commitment depends on a physical interplay between the lipid and serum albumin, which is difficult to reproduce *in vitro* (Kim et al., 2007; Brancucci et al., 2017). Therefore, and notwithstanding the fact that -SerM and -SerM/choline conditions cannot fully simulate *in vivo* conditions either, we decided to use serum-free medium for all experiments that quantify parasite sexual commitment in this study.

Importantly, SCRs determined by this HCI assay are highly consistent with the corresponding gametocyte formation rates observed by light microscopy of Giemsa-stained blood smears prepared three days after the HCI-based readout (**Figure 1E**). In absolute numbers, the NF54/ap2g-mScarlet line showed a mean SCR of 31.7% (95% CI: 27.5-36.9%) under inducing conditions (-SerM) and a baseline SCR of 5.2% (95% CI: 2.5-7.6%) under commitment-repressing conditions (-SerM/choline). To further validate assay performance, we performed choline titration experiments that revealed half-maximal inhibition of sexual

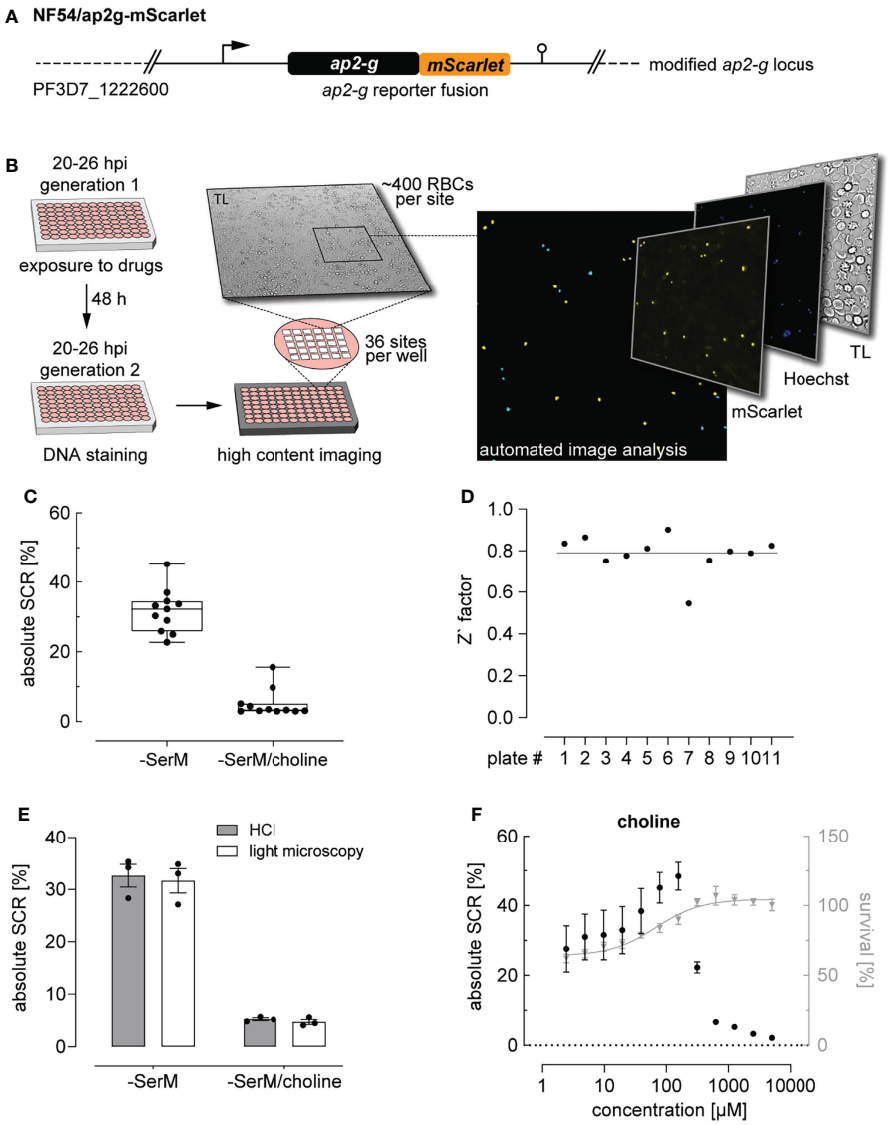

FIGURE 1 | High content imaging-based quantification of SCRs. **(A)** Schematic of the modified *ap2-g* locus in NF54/ap2g-mScarlet parasites. mScarlet, red fluorescent protein. **(B)** Experimental setup of the high content imaging-based sexual commitment assay. TL, transmitted light. **(C)** Rates and variation of sexual commitment in NF54/ap2g-mScarlet parasites under SCR-inducing (-SerM) and inhibiting (-SerM/choline) control conditions. Boxplots show interquartile ranges; whiskers mark minimal and maximal values. Black bullets represent the mean SCRs measured in individual wells (obtained from 6 wells per screening plate) for eleven independent biological replicate experiments. **(D)** Z' factors determined for each screening plate. n=11; the line indicates the mean SCR across all plates. **(E)** SCRs and gametocyte formation rates under SCR-inducing (-SerM) and inhibiting (-SerM/choline) conditions quantified by high content imaging (HCI) and by light microscopy of stage II gametocytes on Giemsa-stained blood smears. n=3, error bars represent the standard error of the mean. **(F)** Dose-response effect of choline on the SCR (black bullets) and parasite survival (grey triangles). Data points represent the mean of three independent biological replicate experiments. Error bars represent the standard error of the mean.

commitment at 275 µM choline (95% CI: 256-293 µM) and impaired parasite multiplication at low choline concentrations, which is consistent with previously published data (Brancucci et al., 2017) (**Figure 1F**).

In summary, these data show that this HCI-based assay allows capturing SCRs under controlled conditions and at a throughput that facilitates the systematic and robust investigation of modulators of parasite sexual commitment.

Many Antimalarials Induce Sexual Commitment at Growth-Limiting Concentrations

We used the above assay to investigate a total of 28 pharmaceuticals for potential effects on sexual commitment in 12-point dose-response assays. In addition to 14 antimalarials, we also screened 14 drugs commonly used in malaria endemic regions, such as analgesics and antihelminthics (**Table 1**).

TABLE 1 | Compounds tested for activities on *P. falciparum* sexual commitment.

Antimalarials	effect on SCR reported in:	Drug class	C_{max}
amodiaquine		quinoline	1.8 μM (Ntale et al., 2009)
atebrin		acridine derivative	125 nM (Croft, 2010)
atovaquone*	(Peatey et al., 2009)	naphtoquinone	68 μM (Baggish and Hill, 2002)
chloroquine*	(Buckling et al., 1997; Buckling et al., 1999a; Buckling et al., 1999b; Peatey et al., 2009)	4-aminoquinoline	500 nM (Mockenhaupt et al., 2000)
dihydroartemisinin	(Portugaliza et al., 2020)	sesquiterpene lactone	7 μM (Saunders et al., 2014)
lumefantrine		arylamine alcohol	530 nM (Ezzet et al., 2000)
mefloquine*	(Peatey et al., 2009)	arylamine alcohol	6 μM (Gutman et al., 2009)
piperaquine	(Peatey et al., 2009)	aminoquinoline	750 nM (Hoglund et al., 2017)
primaquine	(Peatey et al., 2009)	8-aminoquinoline	920 nM (Mello et al., 2018)
proguanil		dihydrofolate reductase inhibitor	870 nM (Wattanagoon et al., 1987)
pyrimethamine*	(Puta and Manyando, 1997)	aminopyrimidine	2 μM (Trenque et al., 2004)
pyronaridine		benzonaphthyridine derivative	550 nM (Jittamala et al., 2015)
quinine*		arylamine alcohol	92 μM (Flanagan et al., 2006)
sulfadoxine	(Puta and Manyando, 1997)	sulfonamide	3.2 μM (Trenque et al., 2004)
Antihelminthics			
albendazole*		benzimidazole	233 nM (Schulz et al., 2019)
ivermectin		avermectin	14.4 nM (Bernigaud et al., 2016)
mebendazole		benzimidazole	1.6 μM (Bekhti, 1985)
moxidectin		pentacyclic lactone	463 nM (Cotreau et al., 2003)
praziquantel		pyrazoniquinoline	2.6 μM (Olliaro et al., 2014)
Antipyretics			
acetylsalicylic acid		COX inhibitor	422 μM (Cerletti et al., 1984)
ibuprofen		nonsteroidal anti-inflammatory drug	40 μM (Mehlisch et al., 2010)
acetaminophen		nonsteroidal anti-inflammatory drug	33.1 μM (Saljoughian, 2016)
diclofenac		nonsteroidal anti-inflammatory drug	51 nM (Miyatake et al., 2009)
Antibiotics			
azithromycin		macrolide	534 nM (Foulds et al., 1990)
doxycycline		tetracycline	3.9 μM (Newton et al., 2005)
Antidiabetics			
gliquidone		sulfonylurea	1.2 μM (von Nicolai et al., 1997)
metformin		guanidine	14.3 μM (Hess et al., 2018)
Steroids			
dexamethasone		glucocorticoid	6 μM (Yang et al., 2008)

Studies that observed an elevating effect of antimalarials on gametocyte production are indicated. Drug classes and the maximum serum concentrations (C_{max}) are shown. Asterisks mark compounds tested in three independent biological replicates.

Compounds showing SCR-inducing effects during primary screening were validated using three independent biological replicate experiments. Dimethyl sulfoxide (DMSO) was used as the vehicle for most compounds and did not show activity on parasite sexual commitment and survival at relevant concentrations (<1%) (**Supplementary Figure 2A**).

While most antimalarials showed a trend towards elevating parasite SCR, this effect was restricted to a narrow concentration window and generally remained linked to drug levels that inhibited asexual parasite replication (**Figure 2A** and **Supplementary Figure 2C**). Chloroquine, pyrimethamine and mefloquine, for instance, had no prominent effect at low concentrations but elevated the parasite SCR just below the IC50 (**Figure 2A** and **Supplementary Figure 2B**). At higher drug concentrations, SCRs fluctuated markedly and reached high values in some instances. However, as these elevated SCRs were observed in populations with complete or near-complete inhibition of parasite survival (**Figure 2A**), they did not result in an enhanced production of sexually committed parasites. In fact, when corrected for parasite survival, only specific sub-IC50 concentrations of the widely used mefloquine and pyrimethamine led to a significant increase in sexual ring stage formation on the absolute scale (**Figure 2B**). While the effect of pyrimethamine was minor, mefloquine, which is known to inhibit protein synthesis *via* direct binding to 80S ribosomes (Wong et al., 2017), increased the formation of ring stage gametocytes by a factor of 2.4 (95% CI: 1.2 to 4.7) at a concentration of 3.9 nM, i.e. several magnitudes below therapeutic concentrations (Karbwang et al., 1987; Gutman et al., 2009). It is conceivable that decreased translation rates in mefloquine treated parasites may serve as a signal for poor nutrient availability, which may in turn act as a cue to invest more resources into increased gametocyte formation in order to maximize host-to-host

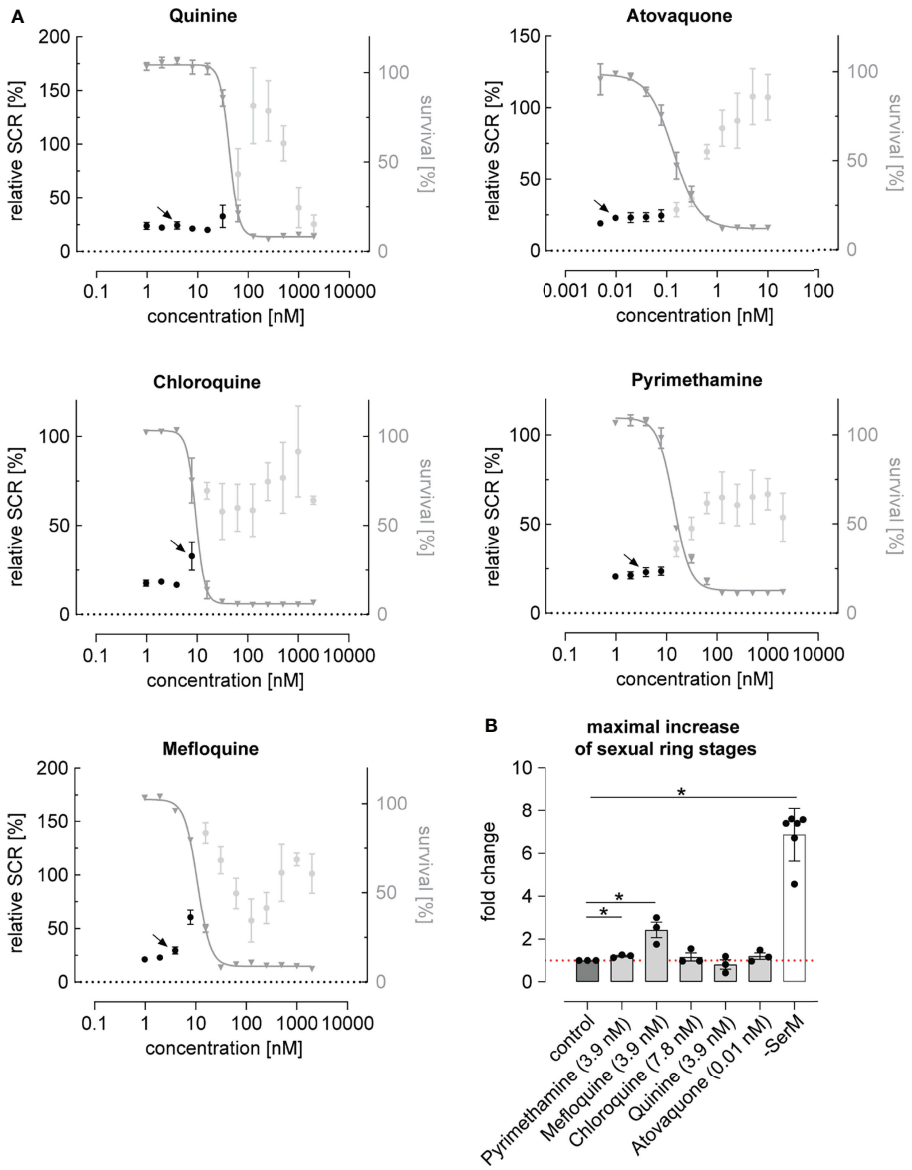

FIGURE 2 | Dose-response relationship between antimalarial compounds and parasite sexual commitment. **(A)** Antimalarials show a general trend towards increasing the proportion of sexually committed parasites at growth-inhibiting concentrations. Mean parasite survival rates and SCRs are indicated by grey triangles and black bullets, respectively. Grey bullets represent SCRs at compound concentrations above the IC50. Values are normalized to the corresponding control conditions (-SerM for SCR) and (-SerM/choline for survival). Data points represent the mean of three independent biological replicate experiments. Error bars represent the standard error of the mean. Arrows indicate data points used for the calculation of SCR fold-changes shown in **(B)**. **(B)** Exposure to sub-therapeutic mefloquine concentrations result in an absolute increase of sexually committed ring stage progeny formed. Pyrimethamine shows a similar but markedly less pronounced effect. Bars indicate mean fold changes of sexual ring stage formation compared to untreated control conditions (-SerM/choline/DMSO), with black bullets representing fold changes from individual biological replicates. Fold changes are defined as $\frac{survial(testcondition)*SCR(testcondition)}{survial(controlcondition)*SCR(controlcondition)}$. For each drug, the results shown are derived from the concentration for which the maximal net increase in absolute sexual ring stage formation was observed. Drug concentrations are indicated. -SerM shows the effect of choline depletion on sexual ring stage formation. Asterisks mark significant differences (p-value < 0.05; paired two-tailed Student's t-test). n=3; error bars represent the standard error of the mean.

transmission under stress conditions (Carter et al., 2013). A possible link between translation inhibition and AP2-G expression, however, remains to be established.

Noteworthy, parasites treated with artemisinin or its derivatives dihydroartemisinin (DHA), artemether and artesunate, emitted autofluorescence at various wavelengths including the TRITC channel, making a fluorescence-based quantification of SCRs impossible. To circumvent this issue and evaluate the effect of DHA, for which a sexual commitment-inducing effect has recently been demonstrated (Portugaliza et al.,

2020), we generated the NF54/ap2g-re9h reporter line (**Figure 3A** and **Supplementary Figure 3**). These parasites express an AP2-G-T2A-RE9H luciferase fusion protein from the endogenous *ap2-g* locus and hence allow using luminescence as a proxy for quantifying AP2-G expression (**Figure 3B**). In contrast to the fluorescence-based assay described above, the NF54/ap2g-re9h line enables determining absolute expression of the AP2-G-RE9H reporter protein in parasite populations per well rather than quantifying SCRs at a single cell level. The NF54/ap2g-re9h cell line showed robustness in reporting sexual commitment under control conditions (Z'-factor of 0.57 (SD: 0.13) as well as the ability to capture the dose-dependent effect of choline on SCRs (half-maximal inhibition of sexual commitment at 194 μM; 95% CI: 136-298 μM) (**Figure 3C**).

After having validated the use of NF54/ap2g-re9h parasites for screening purposes, we made use of this line to quantify the effect of selected drugs on sexual commitment. These experiments confirmed the SCR-inducing effect of mefloquine at sub-therapeutic conditions (**Figure 3D**), and the lack thereof after treatment with atovaquone (**Figure 3E**), corroborating the results obtained with the NF54/ap2g-mScarlet line (**Figure 2A**). Importantly, no increase in SCRs was observed following exposure to DHA (**Figure 3E**).

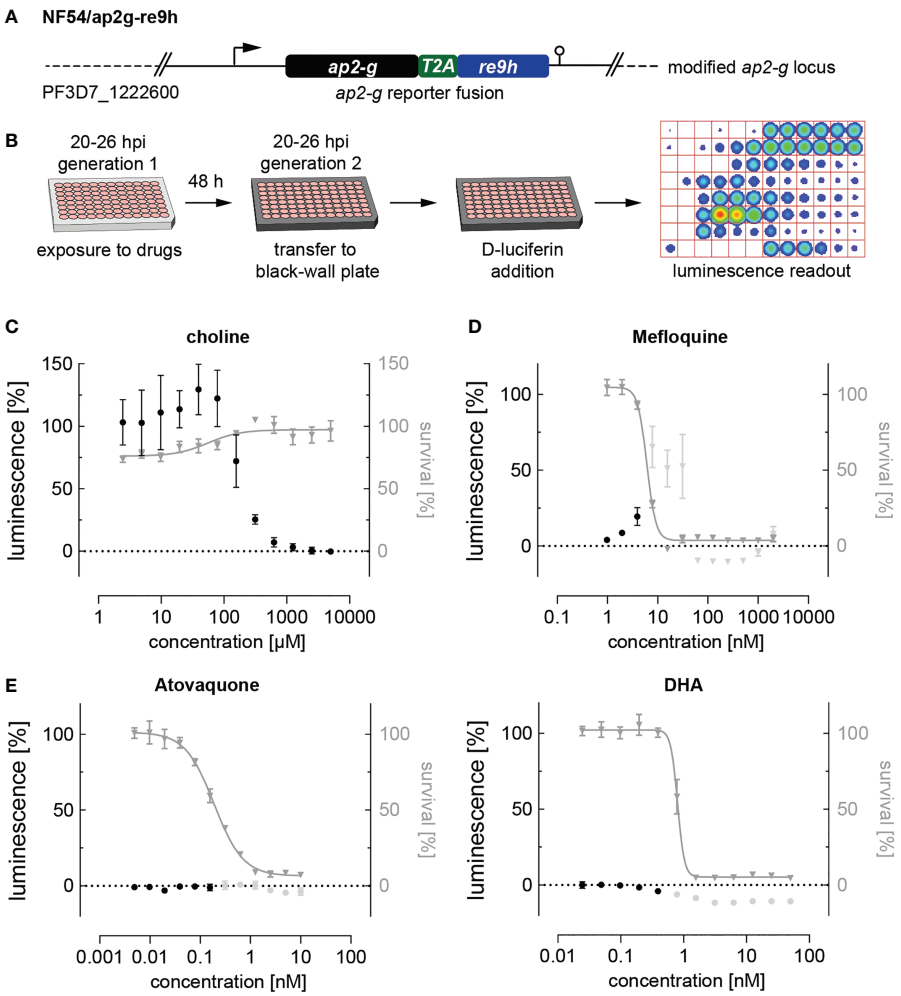

FIGURE 3 | Luciferase-based quantification of sexual commitment. **(A)** Schematic of the modified *ap2-g* locus in NF54/ap2g-re9h parasites. *re9h*, gene encoding red-shifted firefly luciferase RE9H. T2A, self-cleaving peptide. **(B)** Experimental setup of RE9H luciferase-based quantification of SCRs. **(C)** Dose-response effect of choline on the SCR (black bullets) and parasite survival (grey triangles). Data points represent the mean of three independent biological replicate experiments. Error bars represent the standard error of the mean. **(D)** Mefloquine induces sexual commitment within a narrow sub-therapeutic window. Mean parasite survival rates and relative RE9H reporter-mediated luminescence as a surrogate for SCRs are indicated by grey triangles and black bullets, respectively. Grey bullets represent SCRs at compound concentrations above the IC50. Values are normalized to the corresponding control conditions (-SerM for SCR) and (-SerM/choline for survival). n=3; error bars represent the standard error of the mean. **(E)** Neither atovaquone nor DHA induce sexual commitment. Mean parasite survival rates and relative RE9H reporter-mediated luminescence as a surrogate for SCRs are indicated by grey triangles and black bullets, respectively. Grey bullets represent SCRs at compound concentrations above the IC50). Values are normalized to the corresponding control conditions (-SerM for SCR) and (-SerM/choline for survival). n=3; error bars represent the standard error of the mean.

Commonly Prescribed Drugs Have No Relevant Effect on Sexual Commitment

Next, we investigated the effect of a collection of frequently used drugs, including antihelminthics, antibiotics as well as compounds used to treat pain, fever and inflammation (**Table 1**). Similar to what we observed for most antimalarials, antihelminthic drugs, including albendazole, ivermectin and moxidectin, showed trends towards increasing sexual commitment in the NF54/ap2g-mScarlet line (**Figure 4A** and **Supplementary Figure 4**). For most compounds, however, this effect was restricted to parasite growth-inhibiting drug concentrations that are substantially higher than the maximum serum levels observed in patients after standard treatment (Cotreau et al., 2003; Newton et al., 2005; Bernigaud et al., 2016; Saljoughian, 2016; Hess et al., 2018; Schulz et al., 2019). Interestingly, moxidectin, a macrocyclic lactone known to be active against *Plasmodium berghei* mosquito stages (Azevedo et al., 2019), also prevented asexual parasite replication rather effectively (IC50 = 159 nM) (**Figure 4B**). While moxidectin induced sexual commitment at concentrations near the IC50, no net increase in ring stage gametocytes was observed. The tested antipyretics (aspirin, ibuprofen, diclofenac and acetaminophen), antibiotics (azithromycin and doxycycline), antidiabetics (metformin, gliquidone) as well as dexamethasone, a corticosteroid with anti-inflammatory and immunosuppressant properties, had no effects on parasite sexual commitment at

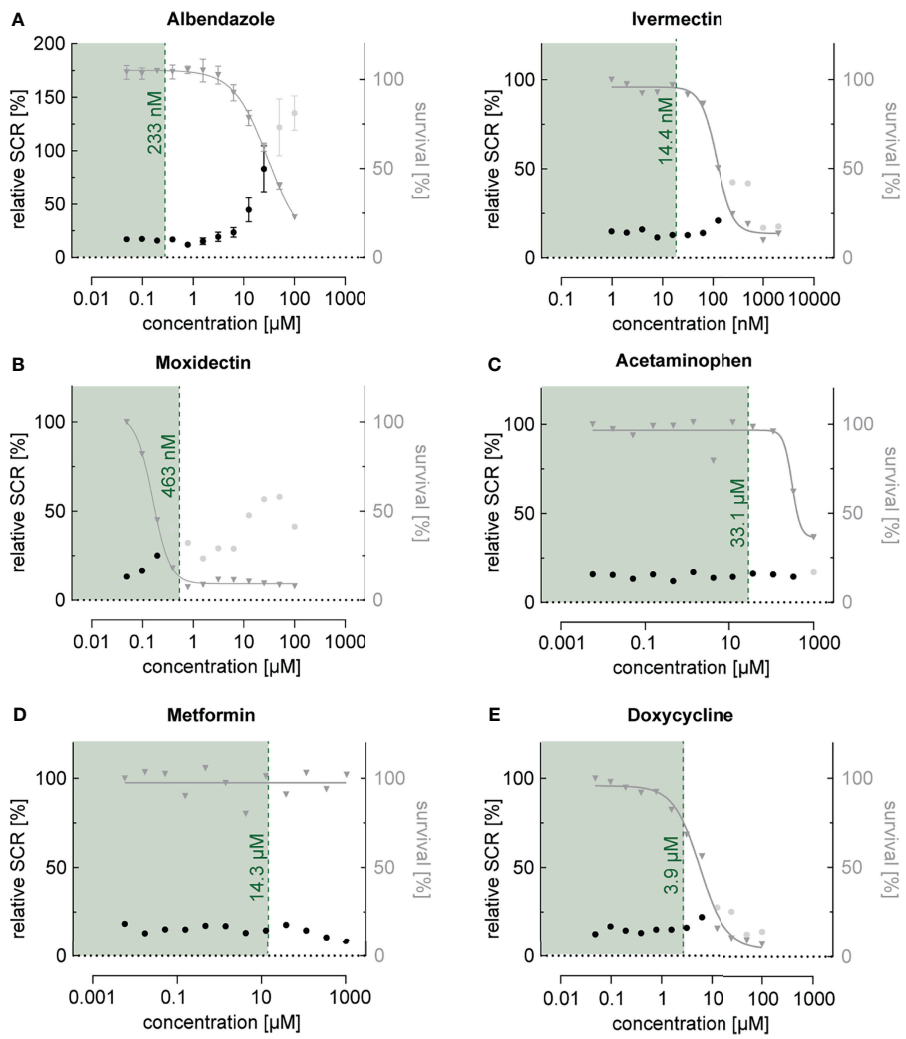

FIGURE 4 | Dose-response relationship between commonly used drugs and parasite sexual commitment. **(A)** The antihelminthics albendazole and ivermectin show a general trend towards induction of sexual commitment at super-physiological concentrations that also affect parasite growth (see also **Figure S4**). **(B)** The antihelminthic moxidectin affects parasite growth as well as SCRs at physiological concentrations. **(C–E)** The commonly used drugs acetaminophen (antipyretic) **(C)**, metformin (antidiabetic) **(D)** and doxycycline (antibiotic) **(E)** have no effect on parasite growth or SCRs at physiologically relevant concentrations. Mean parasite survival rates and SCRs are indicated by grey triangles and black bullets, respectively. Grey bullets represent SCRs at compound concentrations above the IC50. The maximum physiological concentrations are indicated in green. Values are normalized to the corresponding control conditions (-SerM for SCR) and (-SerM/choline for survival). Albendazole: n=3; error bars represent the standard error of the mean. Ivermectin, Moxidectin, Acetaminophen, Metformin and Doxycycline: n=1.

medically relevant concentrations (Cerletti et al., 1984; von Nicolai et al., 1997; Yang et al., 2008; Miyatake et al., 2009) (**Figures 4C–E** and **Supplementary Figure 4**).

Cellular Stress Modulates Sexual Commitment Rather Than Target-Specific Activities

Drug resistance has been associated with increased gametocyte carriage in malaria patients with uncomplicated P. *falciparum* infections (Price et al., 1999; Méndez et al., 2007; White, 2008; Bell et al., 2012). However, whether these observations are a consequence of a higher burden of asexually replicating parasites or higher rates of sexual commitment is unclear. To address this question, we tagged the *ap2-g* locus in the multidrug-resistant *P. falciparum* strain TM90C2B using the same CRISPR/Cas9 gene editing approach employed to generate the NF54/ap2g-mScarlet line (**Figure 5A** and **Supplementary Figure 5**). The TM90C2B strain has reduced susceptibility to chloroquine, cycloguanil, pyrimethamine and atovaquone (Chugh et al., 2015). As expected, transgenic parasites of the TM90C2B/ap2g-mScarlet cell line showed substantially higher tolerance to chloroquine and pyrimethamine compared to the non-resistant NF54/ap2g-mScarlet control line (**Figure 5B**). For chloroquine, the IC50 increased approximately 8-fold from 9.4 nM (95% CI: 8.477 to 10.30 nM) to 71.2 nM (95% CI: 63.79 to 79.69 nM) and for pyrimethamine by a factor of 2`500 from 14.1 nM (95% CI: 12.79 to 15.42 nM) to 36.1 µM (95% CI: 22.9 to 175.9 µM). Similar to the observations made for NF54/ap2g-mScarlet parasites, chloroquine showed a dose-dependent trend towards increasing sexual commitment in the TM90C2B/ap2g-mScarlet line. Importantly, however, drug concentrations that led to increased SCRs in the drug-sensitive NF54/ap2g-mScarlet strain were ineffective in drug-resistant TM90C2B/ap2g-mScarlet parasites. In these multidrug-resistant parasites, increased SCRs were only observed at chloroquine concentrations that inhibited asexual replication beyond the IC50 (**Figure 5B**). Likewise, pyrimethamine, for which we observed a minor but statistically significant inducing effect on the formation of sexual ring stages (see **Figure 2**), elevated sexual commitment in TM90C2B/ap2g-mScarlet cells. Again, this activity was exclusively observed at drug concentrations that substantially inhibited parasite growth (>10 µM) and in this case even exceeded the serum concentrations observed in patients following standard treatment (median of 400 nM (Trenque et al., 2004)).

DISCUSSION

Malaria transmission relies on the formation of gametocytes from a pool of asexually replicating parasites. The proportion of parasites that differentiate into these sexual sages, the so-called sexual commitment rate, is variable and – at least to a certain extent – driven by cues in the microenvironment of intra-erythrocytic parasites (Williams, 1999; Dyer and Day, 2003; Fivelman et al., 2007; Peatey et al., 2009; Brancucci et al., 2015; Brancucci et al., 2017; Portugaliza et al., 2020). While a number of conditions, including exposure to antimalarial drugs, have been suggested or demonstrated to stimulate sexual commitment (Maswoswe et al., 1985; Buckling et al., 1997; Puta and Manyando, 1997; Drakeley et al., 2006; Babiker et al., 2008; Peatey et al., 2009; Baker, 2010;

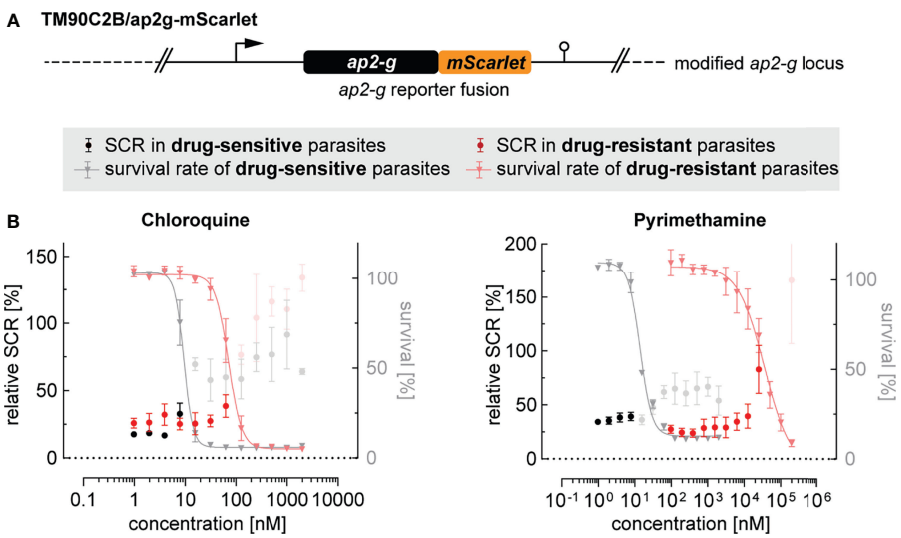

FIGURE 5 | Effect of antimalarials on SCR in drug-resistant parasites. **(A)** Schematic of the modified *ap2-g* locus in multidrug-resistant TM90C2B/ap2g-mScarlet parasites. **(B)** Trends towards increased sexual commitment are linked to growth-inhibiting concentrations of Chloroquine and Pyrimethamine in drug-sensitive as well as in drug-resistant parasite lines. For NF54/ap2g-mScarlet, mean parasite survival rates and SCRs are indicated by grey triangles and black bullets, respectively (values adopted from **Figure 2A**). For drug-resistant TM90C2B/ap2g-mScarlet parasites, mean parasite survival rates and SCRs are indicated by light red triangles and red bullets, respectively. Grey and light red bullets represent SCRs at compound concentrations above the IC50. Values are normalized to the corresponding control conditions (-SerM for SCR) and (-SerM/choline for survival). n=3; error bars represent the standard error of the mean.

Mantel et al., 2013; Regev-Rudzki et al., 2013; Chaubey et al., 2014; Brancucci et al., 2017; Portugaliza et al., 2020), the impact of therapeutic interventions on gametocyte production and malaria transmission is still a matter of debate. Differences in drug-susceptibility between *P. falciparum* asexual parasites and gametocytes, combined with the long period of sexual differentiation, render an evaluation of drug-induced effects on gametocytogenesis a challenging endeavor. Here, we established a high content imaging-based assay to systematically probe the impact of a comprehensive set of antimalarials and other drugs commonly prescribed in malaria-endemic regions on *P. falciparum* sexual commitment. Allowing for the simultaneous quantification of parasite survival and SCRs, this assay facilitated measuring net effects on gametocyte formation.

Consistent with previous observations, we found that *P. falciparum* parasites tend to elevate SCRs following *in vitro* exposure to a multitude of drugs, including, but not limited to antimalarials. While this effect was negligible for most tested compounds, some antimalarials showed a trend towards inducing the rate at which parasites committed to the sexual pathway. Chloroquine for example, elevated the SCR by a factor of 1.64 (95% CI: 0.4-6.1) at a concentration of 7.8 nM (see **Supplementary Figure 2B**) when compared to the untreated control population. This effect, however, was restricted to a narrow drug concentration window around the IC50 value and did not cause a net increase in sexual ring stages formed (see **Figure 2B**).

Contrary to this general trend, exposure to pyrimethamine and mefloquine did not only elevate the SCR but also caused a low to moderate net increase in absolute numbers of sexual ring stages, respectively. These gametocytogenesis-promoting activities were linked to specific concentrations (3.9 nM for pyrimethamine; 3.9 nM for pyrimethamine and mefloquine) near the IC50 for both drugs. While the 2.4-fold increase in sexual ring stages formed (95% CI: 1.2-4.7) after exposure to 3.9 nM mefloquine was the highest activity observed throughout this study, this value is substantially lower compared to the gametocytogenesis-promoting effect observed under LysoPC/choline-depleted -SerM control conditions (fold change of 7.3; 95% CI: 6.1-8.7). Considering this relatively low drug-induced activity, as well as the narrow drug concentration window within which sexual commitment was elevated, it seems highly unlikely that drug treatment *per se* could have a relevant effect on promoting gametocyte production and malaria transmission in real life settings.

Nevertheless, our data reinforce the view that parasites can change rates of sexual commitment and probably also the absolute number of gametocytes formed in response to exposure to drugs at sub-curative levels. Fueled by previous reports about increased gametocytaemias and mosquito infectivity following treatment of parasites with drug resistance mutations (Hogh et al., 1998; Bell et al., 2012), our observations thus raise the question as to whether therapeutic drug concentrations could provoke a disproportionally high rate of gametocyte formation in drug-resistant parasites. Using the multidrug-resistant parasite strain TM90C2B, we could not observe such effects for chloroquine and pyrimethamine and their activities on SCRs remained tightly linked to growth-inhibiting drugs levels. For instance, while we found the TM90C2B parasites to elevate SCRs in response to pyrimethamine exposure, this activity occurred only at a drug concentration of 25 μM, i.e. at growth-inhibiting concentrations close to the IC50 and >2`500-fold higher compared to the concentration that led to elevated SCRs in the pyrimethamine-sensitive NF54 strain.

The strict link between unfavorable growth conditions and elevated parasite SCRs strongly suggests that drug-induced sexual commitment is linked to general stress responses, rather than to compound-specific effects targeting the sexual commitment pathway. In fact, to date we are missing strong evidence for the ability of antimalarials or other drugs to interfere with the molecular process of variable gametocyte formation. Considering the involvement of epigenetic control mechanisms and phospholipid metabolism in the regulation of parasite sexual commitment (Brancucci et al., 2014; Coleman et al., 2014; Brancucci et al., 2017), it would however not be surprising to observe corresponding effects for drugs interfering with these processes specifically. For instance, histone deacetylase inhibitors, which have important applications in anti-cancer treatments (Eckschlager et al., 2017) and show promising activity against *P. falciparum* blood stage parasites (Andrews et al., 2012; Chua et al., 2017), may interfere with heterochromatin-mediated silencing of the *ap2-g* locus. Similarly, choline kinase inhibitors, for which a direct effect on parasite sexual commitment has previously been demonstrated (Brancucci et al., 2017), were proposed as new therapeutic tools against a variety of human diseases, including bacterial and parasitic infections (Lacal et al., 2021). It will thus be important to carefully evaluate potential effects of such molecules on sexual commitment and gametocyte formation before developing them into antimalarial agents.

Based exclusively on *in vitro* experiments, it is clear that the data presented here cannot fully reflect the complex situation found in patients infected with *P. falciparum*. For example, the different microenvironments that parasites encounter at sequestration sites, including the bone marrow and spleen, may have profound effects on drug kinetics and bioavailability. Furthermore, in recent efforts, Portugaliza and colleagues simulated the short *in vivo* half-life of artemisinin/DHA by exposing *in vitro* cultured parasites to short drug pulses and identified stage-specific effects on sexual commitment (Portugaliza et al., 2020). While ring stage populations exposed to 3-hour pulses of DHA responded with decreased SCRs, trophozoites showed elevated SCRs following drug pressure. By contrast, the experiments presented here did not reveal an effect of this artemisinin derivative on parasite SCR. These discrepancies are likely a result of the distinct experimental setups used – particularly the different periods of drug exposure used. Clearly, a comprehensive picture of physiologically relevant links between *P. falciparum* gametocyte production and drug pressure can only be gained by accounting for a variety of parameters including pharmacokinetics, pharmacodynamics as well as different host determinants and microenvironments. The limitations of our *in vitro* studies notwithstanding, the data presented here imply that none of the existing antimalarial drugs act specifically on the molecular pathways controlling sexual

commitment and are hence unlikely to significantly enhance malaria transmission. This is in line with studies reporting that combination therapies, in particular ACTs, are associated with an effective reduction in gametocyte carriage (Okell et al., 2008; Ippolito et al., 2017; WHO., 2020) and indicates that the positive effect of antimalarial treatment clearly outweighs potential risks of increased transmission.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding authors.

AUTHOR CONTRIBUTIONS

BT performed all experiments, analyzed, and interpreted the data. EH, AP, and BT generated the transgenic parasite lines. TB performed experiments performed with NF54/ap2g-re9h parasites. NB and TV conceived of the study, designed and supervised experiments, and provided resources. NB prepared illustrations and wrote the manuscript. TV edited the manuscript. All authors contributed to the final editing of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2022.802341/full#supplementary-material

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REVIEW
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Transmission-Blocking Strategies Against Malaria Parasites During Their Mosquito Stages

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Malaria is still the most widespread parasitic disease and causes the most infections globally. Owing to improvements in sanitary conditions and various intervention measures, including the use of antimalarial drugs, the malaria epidemic in many regions of the world has improved significantly in the past 10 years. However, people living in certain underdeveloped areas are still under threat. Even in some well-controlled areas, the decline in malaria infection rates has stagnated or the rates have rebounded because of the emergence and spread of drug-resistant malaria parasites. Thus, new malaria control methods must be developed. As the spread of the *Plasmodium* parasite is dependent on the part of its life cycle that occurs in mosquitoes, to eliminate the possibility of malaria infections, transmission-blocking strategies against the mosquito stage should be the first choice. In fact, after the gametocyte enters the mosquito body, it undergoes a series of transformation processes over a short period, thus providing numerous potential blocking targets. Many research groups have carried out studies based on targeting the blocking of transmission during the mosquito phase and have achieved excellent results. Meanwhile, the direct killing of mosquitoes could also significantly reduce the probability of malaria infections. Microorganisms that display complex interactions with *Plasmodium*, such as *Wolbachia* and gut flora, have shown observable transmission-blocking potential. These could be used as a biological control strategy and play an important part in blocking the transmission of malaria.

Keywords: malaria, transmission-blocking, drug, transmission-blocking vaccine, *Wolbachia*, mosquito intestinal flora

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1 INTRODUCTION

Malaria is still the most prevalent parasitic disease affecting humans globally, with approximately 228 million cases and 405,000 deaths per year (Ashour and Othman, 2020). Although it has been controlled well in many regions, in some underdeveloped areas, especially in southern Africa, more than one billion people are at risk. The current successes mainly rely on treatment with artemisinin-based combination therapy (ACT), the indoor residual spraying of insecticides, and insecticide-treated mosquito nets. However, existing malaria control measures have become less effective owing

to the emergence of multidrug-resistant parasites and insecticide-resistant mosquitoes, resulting in a recent pause and even a reverse in the reduction of malaria infections (Bhatt et al., 2015; Ippolito et al., 2018; Romoli and Gendrin, 2018; Shaw and Catteruccia, 2019; Moyes et al., 2020). As a warning, it was reported that resistance to frontline artemisinin-based drugs was spreading in the Greater Mekong Subregion of Southeast Asia. Recent studies have shown that similar artemisinin-resistant strains have appeared in Africa, which may be a dangerous signal for global malaria control (Lubell et al., 2014; Slater et al., 2016; Stokes et al., 2021; L'Episcopia et al., 2021; Owoloye et al., 2021). Therefore, to prevent the resurgence of malaria in well-controlled regions and ensure the progressive decline of malaria infection in high-incidence areas, it is necessary to find more effective and safer malaria control strategies.

Thus far, the development of new drugs is still an important method of malaria control, but given the current situation of increasing drug resistance, this method may face huge pitfalls and result in relatively small benefits. Many countries and regions are committed to eradicating malaria, rather than simply controlling its incidence; thus, safe and effective malaria vaccines could be a crucial tool. At present, the development of malaria vaccines mainly consists of three types, targeting different stages of the malaria parasite life cycle: (1) pre-erythrocytic vaccines targeting the sporozoites and liver stages; (2) blood-stage malaria vaccines targeting the asexual blood stages; and (3) transmission-blocking vaccines (TBVs) targeting the sexual stages and mosquito midgut antigens (Carter et al., 2000; Richie and Saul, 2002; Goodman and Draper, 2013). Currently, the pre-erythrocytic vaccines whose main candidate antigen is circumsporozoite protein act to prevent the appearance of the hepatic and erythrocytic stages *via* attacking the sporozoites (Molina-Franky et al., 2020). In addition, the main liver-phase vaccine RTS, S and blood-phase vaccine, whose candidate protein is the merozoite antigen, are mainly designed to prevent the appearance of the erythrocytic stage to provide benefits for clinical malaria (Abdulla et al., 2008; Bejon et al., 2008). Therefore, to block the spread of malaria and eliminate the possibility of malaria infection, TBVs, which mainly target proteins related to the developmental stage after the gametocyte stage (processes that occur in mosquitoes), may be an excellent choice (Wang et al., 2017). In addition, it was predicted that TBV administration could reduce child mortality even in areas of high endemicity (Smith et al., 2001). TBVs can also slow the spread of mutant parasites, prolonging the efficacy of antimalarial drugs and vaccines (Kaslow, 2002). The study of a multi-stage combined vaccine facilitated more extensive application prospects for TBVs (Draper et al., 2018; Yusuf et al., 2019).

However, it is inevitable that the development of vaccines is an extremely long process that requires time to overcome many difficulties and technical obstacles. In fact, several studies have shown that some biological control strategies could also provide solutions to blocking the transmission of malaria (Caragata et al., 2020). Throughout the life cycle of malaria, in addition to the interactions between the malaria parasites and their hosts, many microorganisms also play integral and unique roles in the parasitic life cycle. An increasing number of research groups are exploring how to use the interaction between malaria parasites and various microorganisms to develop malaria control strategies during the growth period (Dahmana and Mediannikov, 2020; Gabrieli et al., 2021). Notably, the technical obstacles of this transmission-blocking strategy may be less difficult to overcome than those of vaccine development, but it is essential to pay great attention to its ecological impact.

In this review, we mainly focus on transmission-blocking drugs, the results arising from the design of TBVs and the challenges that need to be overcome in the subsequent period, as well as summarizing existing biological blocking strategies, which are mainly based around *Wolbachia* and the mosquito intestinal flora. We believe that these findings may provide a reference for the development of malaria transmission-blocking strategies in the future.

2 TRANSMISSION-BLOCKING DRUGS FOR *PLASMODIUM*

Although ACT treatment is very effective in killing the asexual blood stage malaria parasites and curing patients, it cannot completely remove mature gametocytes from the blood. Therefore, malaria patients treated with ACT remain infectious to blood-sucking mosquitoes for 1–3 weeks (WWARN Gametocyte Study Group, 2016). Primaquine is currently the only malaria transmission-blocking drug recommended by the WHO. It can effectively remove mature gametocytes from the blood, but it is not being widely used owing to its safety issues in glucose-6-phosphate dehydrogenase-deficient patients (White et al., 2012). Therefore, in recent years, many studies have been exploring new drugs to solve this problem. Some progress has been made in the improvement of the structure of primaquine itself and in the identification of new possible blocking drugs from natural products derived from plants and microorganisms (Moyo et al., 2020; Boechat et al., 2020).

In addition to killing gametocytes, the spread of malaria could also be blocked by targeting other parasitic stages that occur in mosquitoes (the gametes, zygotes, oocytes, and oocysts) (Smith et al., 2014). Atovaquone and a combination of atovaquone and proguanil were reported to be capable of reducing mosquito infectivity, hence blocking malaria transmission, *via* inhibiting ookinete formation and oocyst maturation (Butcher and Sinden, 2003; Vos et al., 2015; Azevedo et al., 2017). Many other drugs have also been found to have similar properties (Wadi et al., 2019). However, a major problem in blocking the spread of malaria in this way is that the delivery of the drugs is indirect. This means that the drugs must remain in the patient's blood for an extended period at an effective concentration, thus greatly inhibiting the development of this transmission-blocking strategy (Wadi et al., 2018). Moreover, the sporogonic stages (gametes, zygotes, ookinetes, oocysts and sporozoites) themselves are still insufficiently studied as drug targets for now. However, antigens from these stages are being extensively

investigated as targets for TBVs. (Sauerwein and Bousema, 2015; Birkett, 2016).

3 TRANSMISSION-BLOCKING FOR *PLASMODIUM* SEXUAL STAGES

Malaria causes alarming morbidity and mortality in more than 100 countries worldwide. On studying the vaccine development of malaria, it was observed that pre-erythrocytic vaccines can only protect residents in areas of low-endemicity from becoming infected, and blood-stage malaria vaccines have been designed to reduce the severity of the clinical disease (Wang et al., 2017). Thus, TBVs, which aim at stopping the spread of malaria and eliminating the possibility of infection (**Figure 1**), have received increasing attention (Kaslow, 2002).

3.1 Transmission-Blocking From Infected Patients to Mosquitoes

Throughout malaria infection progression, the asexual forms are responsible for clinical malaria, while the sexual stages are responsible for continued transmission *via* mosquitoes. In the erythrocytic stage, < 1% of *Plasmodium* parasites commit to forming gametocytes in infected red blood cells. After male and female gametocytes are ingested in a mosquito blood meal, they break out of their red blood cells and initiate the sexual stage of parasite development. Therefore, from a life history perspective, blocking malaria gametocytes from transferring to mosquitoes is the first important step in reducing global malaria transmission.

So far, most of the gametocyte surface proteins considered as TBV candidates have been from the six-cysteine motif (6-CM) protein family, and the most studied members of this family are Pfs48/45 and Pfs230 (Saul, 2007). Knockout experiments have demonstrated that these two proteins are essential for fertilization after the gametocyte enters the mosquito (van Dijk et al., 2001; Eksi et al., 2006). Although antibodies targeting Pfs230 could prevent oocyst formation in mosquito through standard membrane-feeding tests and/or direct membrane-feeding tests, the applied research seems to have stagnated because the functional antibodies used for the investigation can only be produced *in vivo* now (Farrance et al., 2011; Miura et al., 2013; Kapulu et al., 2015). Producing recombinant Pfs230 proteins *in vitro* remains challenging. This is mainly because Pfs230 is a >300 kDa protein consisting of 14 CM domains and both the size and cysteine-rich nature of the molecule have hampered its production as an intact protein. Therefore, researchers have been attempting to determine the domain that is responsible for the blocking ability of this protein for future vaccine development. A study produced multiple fragments of the Pfs230 molecule using a eukaryotic wheat germ cell-free expression system. In subsequent experiments, they found that protein fragments containing CM domain 1 displayed strong transmission-blocking effects, while antibodies

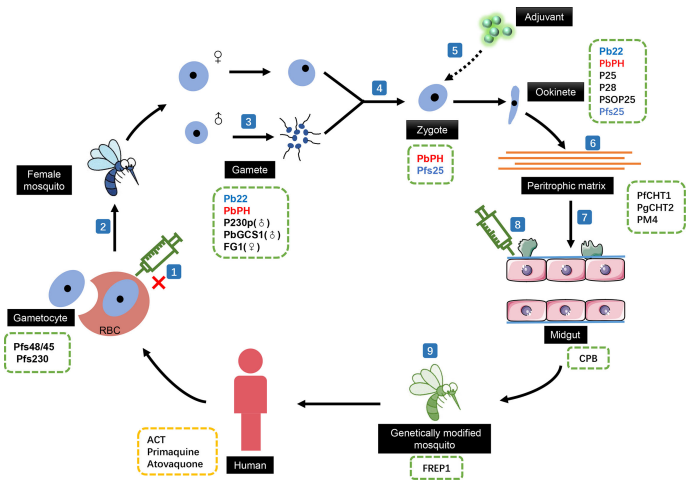

FIGURE 1 | The effect of transmission-blocking vaccines (TBVs) in the sexual reproductive stages of malaria. (1) TBV cannot act on gametocytes that have not escaped from red blood cells. The development of TBVs based on the (2) surface proteins of the gametocytes, (3) male gamete-related targets, and (4) fertilization process-related proteins can effectively block the development process of malaria in mosquito. (5) The lack of inherent antigenicity means that TBVs based on ookinete surface proteins need adjuvants in actual use. Blocking the ability of motility ookinetes to break through the (6) peritrophic matrix and the (7) physical and immune barriers of the midgut to prevent its colonization in the midgut is another development direction of TBVs. (8) TBVs targeting mosquito midgut proteins can also effectively block ookinete colonization. (9) Directly genetically modified mosquitoes to prevent the development of ookinetes. Candidate target proteins for transmission blocking antibodies in different stages of malaria life cycle were listed in green dotted boxes. Anti-malaria drugs that could influence the transmission were listed in yellow dotted box. ACT, artemisinin-based combination therapy; Pfs48/45, *Plasmodium falciparum* gametocyte surface protein; Pfs230, *P. falciparum* gametocyte surface antigen; Pb22, a conserved protein (PBANKA_0305900) in *Plasmodium berghei*; PbPH, *P. berghei* pleckstrin homology gene; PbGCS1, *P. berghei* Generative Cell Specific 1; FG1,female gamete peptide 1; Pfs25, *P. falciparum* sexual-stage surface protein; P25, the major surface proteins of *Plasmodium* ookinetes; P28, Ookinete surface antigen-like protein; PSOP25, putative secreted ookinete protein 25; PfCHT1, *P. falciparum* chitinase; PgCHT2, *P. gallinaceum* chitinase; PM4, plasmepsin 4; CPB, Carboxypeptidase B; FREP1, Fibrinogen-related protein 1.

generated using constructs without CM domain 1 showed no inhibition. This suggests that CM domain 1 may be an excellent choice (Tachibana et al., 2019). In addition, studies have reported that liposome vaccine adjuvant admixed with Pfs230 fragments could trigger a stronger blocking effect (Huang et al., 2020). Similarly, although the molecular size of Pfs48/45 is appropriate, the correct and efficient production of recombinant vaccines is still an urgent problem that needs to be solved. Multiple studies tackling this issue have also achieved good results, either using DNA plasmid encoding after codon optimization or endo H enzymatic deglycosylation in plants. Both significantly enhanced the immunogenicity of recombinant Pfs48/45 and strengthened its transmission-blocking ability (Datta et al., 2017; Mamedov et al., 2019; Lee et al., 2020). In addition, a study reported that a Pfs230–Pfs48/45 chimeric malaria transmission-blocking vaccine could induce a stronger blocking effect than a single protein and identified a modified construct (ProC6C) as a possible solution to the low yield of this vaccine (Singh et al., 2020; Singh et al., 2021).

In addition, this vaccine is unusual in that the antibodies cannot kill the gametocytes in the red blood cells of malaria patients; it is only during the mosquito's blood meal from an infected host that the antibodies can efficiently act on the gametes after they have emerged from their host red blood cells, thus preventing infection in the mosquito and halting the spread of malaria (Carter and Chen, 1976; Gwadz, 1976; Saul, 2007). In the Makoni district of Zimbabwe (an area of low to modest malaria transmission), enzyme-linked immunosorbent assay revealed the prevalence (64% positivity at 1:500 dilution in 66 randomly selected plasma samples) of antibodies against recombinant Pfs48/45 (mean A405 nm = 0.53, CI = 0.46–0.60) and Pfs47 (mean A405 nm = 0.91, CI = 0.80–1.02), antigens specific to the sexual stages of the parasite. A mosquito membrane-feeding test demonstrated that samples positive for the Pfs48/45 antibody in the enzyme-linked immunosorbent assay had the ability to reduce malaria transmission (Paul et al., 2016). These results indicate that there may be considerable natural immune antibodies in the infected population that play a role in transmission-blocking, resulting in a low local prevalence. Therefore, identifying the antibodies against gametocyte-expressed proteins produced by natural immunity may accelerate the progress of vaccine development.

Furthermore, intact mature gametocytes do not undergo phagocytosis mediated by monocytes, benefiting malaria transmission. However, red blood cells containing gametocytes that are not transmitted to mosquitoes during infection will die and then be eliminated by phagocytic cells. Therefore, the analysis of the molecular mechanisms in this phagocytic process and subsequent immune response may help to identify more targets for transmission-blocking strategies (Bansal et al., 2016).

3.2 Transmission-Blocking for Gametocytes Fertilized Into Zygotes

When gametocytes are ingested by mosquitoes, they transform into gametes as the environment changes (Kehrer et al., 2016). Each female gametocyte forms a single immotile macrogamete, while male gametocytes generate up to eight flagella-like microgametes in a process called exflagellation. Subsequently, the fully differentiated gametes need to dissolve the inner parasitophorous vacuole membrane and the outer erythrocyte membrane to form diploid zygotes, which then continue to develop and mature in mosquitoes (Paul et al., 2002; Sologub et al., 2011; Deligianni et al., 2013). In general, the fertilization process lacks the protection of the host cell membrane and involves many cells and biochemical processes, providing plenty of potential drug targets (Sinden et al., 2012). Therefore, malaria-blocking strategies aimed at the fertilization process have received increasing attention.

A recent mathematical model analysis showed that the human antibodies ingested by mosquitoes were effective at inhibiting fertilization in mosquito midgut, resulting in a decrease in the density of oocysts (Teboh-Ewungkem et al., 2021). This indicates that antibodies targeting gamete proteins may be a potential strategy for transmission-blocking. While female gametocytes only have to leave their red blood cells to become gametes, male gametogenesis also includes three rounds of mitosis and flagellum construction to produce eight gametes (Schall, 2000; Reece et al., 2008). Thus, male gamete-related targets have become the focus of TBV development. A conserved protein of 22 kDa in *Plasmodium berghei*, named Pb22, is located on the plasma membrane of gametes and ookinetes during gamete-to-ookinete development. A recent study showed that the exflagellation of male gametes (~89%) and ookinete numbers (~97%) were significantly reduced after Pb22 was knocked out, and these defects were rescued in parasites when Pb22 was restored. Further analysis showed that the defects of the Pb22 knockout (KO)line were limited to the male gametes, and the female gametes in Pb22-KO line were fertile at the wild-type level, indicating that Pb22 was indeed a key protein in the development of male gametes. Of the Pb22-KO line male gametocytes, 30% failed to assemble axonemes, whereas ~ 48.9% formed flagella but failed to egress from the host erythrocytes (Liu et al., 2021). In addition, antibodies against the highly conserved *Plasmodium berghei* pleckstrin homology gene PbPH that is localized on the surface of gametes, zygotes, and ookinetes significantly inhibited *the* exflagellation of male gametocytes and the formation of ookinetes in a concentration-dependent manner (Kou et al., 2016). P230p, which is expressed only in male gametocytes and gametes, is another potential target. Studies have shown that, after knocking out P230p, male gametes can normally form outer flagella but cannot attach to the red blood cell membrane, resulting in a significant reduction in zygotes and oocysts (Marin-Mogollon et al., 2018). Moreover, it was previously observed that the knockout of Generative Cell Specific 1 (GCS1) in angiosperms could cause male sterility, while the knockout of PbGCS1 in *Plasmodium* also showed a male sterility phenotype (Hirai et al., 2008). Subsequent studies further demonstrated that GCS1 (also known as Hap2) was indeed a TBV target candidate; TBVs based on GCS1 could trigger a strong transmission-blocking effect (Blagborough and Sinden, 2009; Angrisano et al., 2017; Qiu

et al., 2020; Feng et al., 2021). This suggests that research across different species may have inherent connections, which is worthy of in-depth exploration.

Importantly, although male gametes have occupied an important position in the development of TBVs, remarkably little is known about the ecology and behavior of male gametes. Male gametes need to locate and fertilize females in the challenging environment of the mosquito blood meal, and studies have shown that tryptophan metabolites can affect the fertilization process. Meanwhile, a proteomic analysis revealed that glycolysis may be the exclusive energy source for the flagellar beat of male gametes (Talman et al., 2014). Moreover, male gametes may not move randomly, and somehow the female gametes attract the male gametes (Carter et al., 2016). At present, studies on this aspect are rare, but further analysis of the factors affecting the fertilization process, would greatly broaden our understanding and facilitate the development of TBVs.

In fact, aside from male gametes, there are other potential vaccine targets. Although a variety of TBV candidates have been discovered from the study of male gametes, the proteins that interact with these targets are still poorly understood. It has recently been found that the combination of female gamete peptide 1, a peptide that binds specifically to the surface of female gametes, and female gametes interferes with the fertilization of male gametes, thereby significantly reducing the number of oocysts (Vega-Rodriguez et al., 2015).

However, the strategy of blocking the spread of malaria during the fertilization process still has an aspect that cannot be ignored. Studies have shown that *Plasmodium* responds to external transmission pressure by increasing the number of male and female gametocytes. When the density of gametocytes is low, *Plasmodium* can increase the rate of transmission success by increasing the proportion of male gametes (Mitri et al., 2009; Ramiro et al., 2011). This fact is of concern as the TBVs targeting the fertilization process almost all target the male gametes, and the *Plasmodium* may offset the effect of these TBVs by increasing the number of male gametes. Therefore, sex-specific vaccines targeting gametes cannot provide satisfactory blocking effects. An understanding of how the malaria parasite senses external pressure is needed and the mechanism of changing gamete distribution is a key issue that needs to be resolved.

4 TRANSMISSION-BLOCKING FOR THE POST-MIDGUT DEVELOPMENTAL STAGE

When female macrogametes are fertilized and transformed into motile ookinetes, they need to invade the midgut epithelium of the mosquito. Upon reaching the basal lamina, the motile ookinetes continue to develop into oocysts. Thousands of sporozoites then form in the mature oocysts, enter the hemocoel and invade the salivary glands of the mosquito in preparation for inoculation into new human hosts (Sinden, 1999). This final process of the reproductive development stage of *Plasmodium* is thought to be another critical step. Transmission-blocking strategies targeting this stage have also received significant attention.

The most common transmission-blocking strategy is inhibiting the ability of the motile ookinetes to directly invade the midgut epithelium. In fact, this invasion process is the most difficult step for development of malaria parasites in mosquito because the motile ookinetes need to break through many obstacles for successful colonization. The peritrophic matrix, composed of proteins, glycoproteins, proteoglycans, and chitin, is the first physical barrier faced by the ookinete (Sieber et al., 1991; Moskalyk et al., 1996; Shen and Jacobs-Lorena, 1998). Antibodies targeting chitinase (PfCHT1 or PgCHT2) and a secreting plasma protease (aspartic protease plasmepsin 4, PM4) in *Plasmodium* have been shown to inhibit the passage of ookinetes through the peritrophic matrix, thereby significantly reducing the number of oocysts and the infectivity of malaria (Li et al., 2005; Li et al., 2010). After the peritrophic matrix, motile ookinetes also need to overcome the physical barrier and innate immune defense system of the midgut epithelium (Meister et al., 2005). The highly conserved ookinete surface proteins (P25 and P28) and the putative secreted ookinete protein 25 have been shown to assist the motility of ookinetes that attach and invade the midgut epithelium. Studies have further shown that antibodies targeting these proteins can affect ookinete maturation and oocyst formation in a concentration-dependent manner (Baton and Ranford-Cartwright, 2005; Zheng et al., 2017). However, it should be noted that the lack of inherent antigenicity of Pfs25 leads to the need for a strong human-use-compatible adjuvant for this vaccine in practical application. In a phase I clinical trial, erythema nodosum associated with a Montanide ISA 51 oil-in-water adjuvant was tested in two cases, and the subjects showed frequent local reactogenicity throughout the trial (Wu et al., 2008). Therefore, the development of safer and more efficient adjuvants is urgently needed for this vaccine. A previous study explored the blocking efficiency of alga-produced Pfs25 in combination with four different human-compatible adjuvants (alum, Toll-like receptor 4 agonist glucopyranosal lipid A plus alum, squalene-oil-in-water emulsion, and glucopyranosal lipid A plus squalene-oil-in-water emulsion) and demonstrated that Toll-like receptor 4 agonist in a squalene-oil-in-water emulsion may be a promising adjuvant (Patra et al., 2015). Moreover, the TatD-like DNase of *Plasmodium*, which is considered a conservative protein that plays an important role in immune escape in the asexual stage, is not only expressed during the red blood cell stage, but also throughout the developmental stages of mosquito vectors (Marin-Esteban et al., 2012). Interestingly, the combined immunization of recombinant TatD-like DNase and Montanide ISA51 was found to induce a strong humoral response and weaken the ability of the malaria parasite to break through the midgut innate immune barrier, which significantly prevented the development and transformation of parasites in the midgut of mosquitoes in a mouse model (Wang et al., 2018).

However, vaccines targeting the protein in the reproductive period of *Plasmodium* still have a disadvantage that cannot be ignored. With the use of these vaccines, the malaria parasites are under great selective pressure; thus, they are likely to produce unexpected mutations and escape immune responses (Coutinho-Abreu and Ramalho-Ortigao, 2010). As the developmental stage is also completed under the influence of the mosquito's system, designs based on mosquito target proteins are a potential method

of solving this problem, and some excellent research results have been reported. The design of vaccines based on midgut surface-related proteins is the main exploration direction. Carboxypeptidase B in the midgut of mosquitoes has been demonstrated to be essential for the sexual development of *Plasmodium* in mosquitoes, and several drugs from the Food and Drug Administration, such as NSC-1014, NSC 332670, and aminopterin, have been confirmed to significantly reduce carboxypeptidase B activity (Mongkol et al., 2015). Further improvements to these three drugs may help in the development of TBVs. In addition, antibodies against some conserved proteins in the midgut microvilli also disrupt the development of *Plasmodium* oocysts (Dinglasan et al., 2007; Lecona-Valera et al., 2016).

It is even more surprising that the direct genetic modification of mosquito vectors may be a more promising solution. A recent study has attempted this approach. Fibrinogen-related protein 1 (FREP1), a member of the fibrinogen-related protein family (also known as fibrinogen domain immunolectin), has been shown to be involved in the infection process of *Plasmodium* in mosquitoes (Dong et al., 2006; Dong and Dimopoulos, 2009; Simoes et al., 2017). Previous studies have shown that FREP1 mainly plays a role in the midgut development stage of *Plasmodium* in mosquitoes (Zhang et al., 2015). In this study, researchers used the CRISPR/Cas9 model to simulate mosquitoes lacking FREP1, and the number of oocysts was indeed significantly suppressed in this phenotype when further infected with *Plasmodium*. However, although the exclusion of FREP1 had an excellent blocking effect on the spread of malaria parasites, many issues still need to be resolved. In subsequent observations, the inactivation of FREP1 had a significant detrimental effect on the health of the mosquitoes, including a significantly lower propensity for blood-sucking, lower fecundity and egg hatching rates, retarded pupation times, and reduced longevity after a blood meal (Dong et al., 2018). This suggests that a target gene with lower detrimental health effects may be needed in the future to ensure the survival of the modified mosquitoes.

In particular, the exploration into blocking strategies during this stage need to consider a particular problem. A recent study showed that when the intensity of mosquito infection was extremely high, starvation conditions reduced the activity of RNA polymerase III in the oocysts, leading to a decrease in the growth and maturation rate of the oocysts. However, when the mosquitoes were given another blood meal, the number of oocysts was completely restored (Habtewold et al., 2021). As the mosquitoes were in a highly infected state during the experiment to evaluate the transmission-blocking effect, these results hint that the change of oocysts number after another feeding might be considered another key assessment factor for the realistic transmission-blocking ability of TBVs.

5 TRANSMISSION-BLOCKING FOR MULTI-STAGE COMBINATION VACCINE

To date, the development of malaria vaccines has been mainly based on a single antigen from different life stages of the malaria parasite. This has raised concerns that single-stage vaccines may be ineffective owing to sequence variability among different parasite isolates, host genetic restrictions of immune responses to specific epitopes, and the short-lived protective immunity induced by some single-antigen vaccines (Shi et al., 2000). Therefore, a multi-stage target combined vaccine is considered a powerful solution. A dual-target vaccine for the asexual and sexual stages has been shown to be feasible; when a fusion protein comprising the *Plasmodium vivax* circumsporozoite and P25 proteins was included in a combination vaccine, significant protective effects (43%) and blocking effects (82%) were observed simultaneously (Mizutani et al., 2014). In addition, several benzimidazole derivative compounds and a series of newly synthesized internal peroxy compounds also exhibited dual effects in the asexual and sexual stages of *Plasmodium* (Miranda et al., 2014; Leshabane et al., 2021). However, combination immunization vaccines targeting different developmental stages in the mosquito phase did not seem to produce synergistic effects. In a previous study, researchers tested the effect of TBVs against both the pre-fertilization antigen Pys48/45 and the post-fertilization antigen Pys25. The results showed that the blocking effect of the composite vaccine was stronger than that of the vaccine based on the Pys48/45 target, but significantly weaker than that of vaccines based on the Pys25 target (Zheng et al., 2016). This suggests that the multi-targeted TBV vaccine may interfere with the induction of antigen-specific antibody responses, and follow-up studies are needed to further explore the reasons for this phenomenon.

6 ENDOSYMBIOSIS IN TRANSMISSION-BLOCKING OF MALARIA

Identifying potential targets based on the life stages of malaria parasites in mosquitoes to design corresponding single target or combination vaccines is an important transmission-blocking strategy. However, this involves the identification of vaccine targets, the determination of vaccine production methods, the correct folding of the corresponding proteins, and the search for relevant vaccine adjuvants. This is a long-term process that requires the long-term delivery of a large workforce and considerable material resources. It is worth noting that there are many endosymbionts naturally present in mosquitoes, and there are also significant differences in malaria parasite infections between different mosquito populations and even regions (Caragata et al., 2020). Therefore, we may be able to directly use these endosymbionts to develop malaria-blocking strategies, which would be extremely beneficial for some current malaria-prone areas. Although there are still some problems to be solved, we believe that this biological blocking strategy would be an important auxiliary strategy to help stop the spread of malaria.

6.1 *Wolbachia*

6.1.1 Transmission-Blocking Strategies

Wolbachia, as a potential bio-replacement strategy, has received significant attention in the control of malaria in recent years

(Dahmana and Mediannikov, 2020). This is mainly owing to the benefits of *Wolbachia* infections, which induce mosquitoes to produce two desirable properties for disease vector control, cytoplasmic incompatibility (CI) and pathogen inhibition (Walker et al., 2021). Meanwhile, there is increasing evidence that *Wolbachia* infections in mosquitoes are common (Baldini et al., 2014; Niang et al., 2018; Jeffries et al., 2021); 25 species of African *Anopheles* mosquitoes have been found to carry 16 varieties of *Wolbachia* infections (Ayala et al., 2019). The desirable induction properties and diverse flora have prompted researchers to further increase their interest in using *Wolbachia* for developing biological control strategies against malaria transmission (**Figure 2**).

Overall, there are two main biological control strategies based on *Wolbachia*: (1) using CI to kill mosquitoes and (2) relying on the transmission-blocking ability of *Wolbachia* in the mosquito stage of *Plasmodium* development. Through CI, *Wolbachia*-infected females produce viable *Wolbachia*-infected offspring when they mate with uninfected males or males infected with the same *Wolbachia* strain. However, *Wolbachia*-infected males only produce viable offspring when they mate with females infected with the same *Wolbachia* strain. Meanwhile, the molecular mechanism of CI has been recently elucidated: CI causes embryonic death when a male expressing the prophage WO genes, *cifA* and *cifB*, mates with an uninfected female or a female infected with an incompatible *Wolbachia* strain. In contrast, embryonic development could be rescued in mosquito females harboring a compatible *cifA*-expressing strain (LePage et al., 2017; Shropshire et al., 2021). In the last five years, based on these properties, various research groups have explored whether they can release a large number of *Wolbachia*-infected male mosquitoes to prevent egg hatching, resulting in adult mosquito numbers being significantly reduced in the target area (Mains et al., 2016; Kittayapong et al., 2018; Kittayapong et al., 2019; Mains et al., 2019; Zheng et al., 2019). Surprisingly, all experiments acquired positive results, providing good research models for subsequent studies.

However, before promoting the CI-based mosquito population control strategy, several key issues need to be resolved. The first is the costs associated with rearing and releasing large number of mosquitoes. The second is that the target population may recover over time after release is halted (Caragata et al., 2020). These pitfalls may need to be dealt with using cost–benefit calculations and sophisticated monitoring. More critically, there is no guarantee that there would be no female mosquitoes released along with the males; thus, there are concerns that accidentally released female mosquitoes may quickly replace the mosquito population in the target area, rendering future suppression releases less effective. Recent studies have attempted to overcome this issue by using aspects of the sterile insect technique during mosquito rearing, with pupae irradiation before release, and positive results have been obtained (Kittayapong et al., 2018; Moretti et al., 2018a; Moretti et al., 2018b; Zheng et al., 2019). However, more efficient male and female separation technology is the focus of further research. In addition, to obtain the most benefit, the timing of the release of the mosquitoes may also need to be considered, although there have been fewer studies along these lines (Huang et al., 2020).

Wolbachia itself also has certain transmission-blocking effects on the malaria parasite. It has been demonstrated to have a strong harmful effect on sporozoites and could significantly reduce the number of sporozoites in the mosquito stage (Gomes et al., 2017). The current understanding is that this effect may involve the influence of *Wolbachia* on the mosquito immune system, nutritional competition, mosquito lifespan, and its interaction with the mosquito microbiome (Kambris et al., 2009; McMeniman et al., 2009; Moreira et al., 2009; Audsley et al., 2018). Studies have shown that anti-*Plasmodium* immune

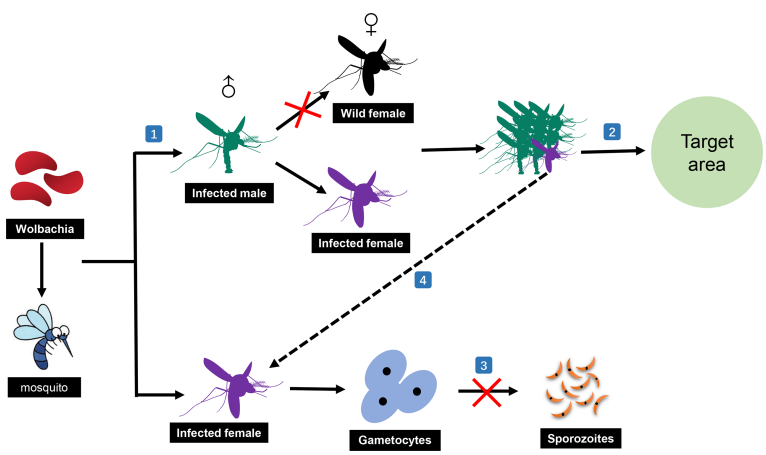

FIGURE 2 | Malaria transmission-blocking strategies based on *Wolbachia*. (1) Male mosquitoes infected with *Wolbachia* can only mate with female mosquitoes infected with the same *Wolbachia* to complete normal reproduction. (2) Numerous infected male mosquitoes can be released in the target area to kill the mosquitoes in the area and thus stop the spread of malaria. (3) *Wolbachia* can also prevent the normal sporozoites development of *Plasmodium* in mosquitoes. (4) Infected female mosquitoes accidentally released with the numerous infected male mosquitoes can still block the spread of malaria through the pathogen suppression ability of *Wolbachia* itself.

genes, including TEP1, LRIM1, Toll pathway gene Rel1, and the effector Defensin 1, were induced by *Wolbachia* in *Anopheles stephensi* mosquitoes (Joshi et al., 2017). However, another study on *Aedes fluviatilis* revealed that the immune activation effects did not seem to include changes in Toll or IMD immune gene transcription (Caragata et al., 2017). Thus, accidentally released female mosquitoes would not be useless. This is another strategy that uses *Wolbachia* to block malaria in mosquitoes. The advantage of using this strategy is that it requires a relatively small number of mosquitoes to replace the original population in the target area owing to the breeding advantage of the new mosquitoes (O'Neill et al., 2018; Zélé et al., 2018; Ryan et al., 2019). However, it is still necessary to consider the bite problem of female mosquitoes and the possible consequences. Although there have been no reports of using this strategy to block the spread of malaria, good progress has been made in blocking dengue fever (Nazni et al., 2019; Ryan et al., 2019). The identification of the actual effect of this strategy in blocking the transmission of malaria requires follow-up studies.

6.1.2 Restrictions on Actual Application

When using *Wolbachia*-infected mosquitoes to develop malaria transmission-blocking strategies, the most critical issue is that the symbiont should establish a stable relationship with the insect carrier, that is, long-term effective vertical or horizontal transmission (Bian et al., 2013; Wilke and Marrelli, 2015; Walker et al., 2021). Although some stable *Wolbachia*-infected mosquitoes have been established through embryonic injection followed by transgenerational spread (Dobson et al., 1999; Sinkins, 2004; Bourtzis et al., 2014), it is still necessary to understand how *Wolbachia* establishes a symbiotic relationship with mosquitoes to develop a more efficient and stable infection plan.

The use of innate immunity to establish a stable symbiotic relationship with mosquitoes is an important mechanism of *Wolbachia* infection. Generally, when *Wolbachia*-free insects are artificially infected, an antimicrobial immune response is triggered to eliminate *Wolbachia*. However, *Wolbachia* could prevent this elimination by evading the AMP immune response or suppressing autophagy-related immune defenses (Zug and Hammerstein, 2015). Meanwhile, another study showed that inhibiting the IMD pathway alone or simultaneously inhibiting the Toll and IMD pathways by RNA interference could significantly reduce the *Wolbachia* load in mosquitoes, although the specific mechanism still needs to be clarified (Pan et al., 2018).

In addition to regulating the mosquito's immune system, the complex interaction between *Wolbachia* and the mosquito's natural microbiota is another aspect of the mechanisms underlying the stability of this infection. Certain species of mosquitoes are not naturally infected with *Wolbachia*. When a *Wolbachia* infection is introduced artificially, it is unsurprising that significant vertical transmission barriers are observed, making it difficult for this infection to spread to the next generation. However, when antibiotics continue to be used to treat these mosquitoes, a significant increase in the titer of *Wolbachia* infection was observed, thus achieving perfect maternal vertical transmission (Hughes et al., 2014). Subsequent studies have also shown that some bacterial groups were negatively related to *Wolbachia* infections in mosquitoes, and Asian symbionts have been experimentally confirmed to limit the vertical transmission of *Wolbachia* in mosquito populations (especially infection in the reproductive tissues) (Hughes et al., 2014; Rossi et al., 2015; Straub et al., 2020). Moreover, it is worth noting that the invasion of malaria parasites into mosquitoes would also affect the bacterial composition in the mosquito's midgut (Sharma et al., 2020), suggesting that related research on *Wolbachia* may deepen our understanding of malaria and thus promote the development of transmission-blocking strategies.

Meanwhile, as the antimalarial ability of *Wolbachia* is controlled by itself but not manipulated by humans; apart from understanding how it infects parasites, we also need to explore whether pathogen escape occurs under infection conditions. To date, although no evidence of pathogen escape was found in wild populations of *Wolbachia*, pathogens seem to be able to survive under infection by certain laboratory strains, suggesting that we need to remain vigilant and take precautions (Pimentel et al., 2021).

The last thing that may require attention is how to determine the presence of *Wolbachia* infections in mosquitoes. At present, most studies use nested PCR tests to detect infections; however, they are limited to amplifying only a few genes (especially 16S rRNA). This is problematic given the possibility of amplifying prokaryotic 16S rRNA genes from non-living cells (Carini et al., 2017; Chrostek and Gerth, 2019). Therefore, more robust evidence is required to determine whether *Wolbachia* strains are established as endosymbionts in *Anopheles* species (Chrostek and Gerth, 2019). Several recent studies have confirmed the existence of natural infection by observing *Wolbachia* infection in the ovaries of mosquitoes over several generations (Ross and Hoffmann, 2021; Walker et al., 2021), but this method is less efficient and is not suitable for vigorous promotion. Thus, a more efficient and convenient method is still urgently needed. Furthermore, studies have shown that different types of *Wolbachia* may have different effects on malaria parasite infection in mosquitoes. It has been reported that infection with certain strains of *Wolbachia* can increase malaria parasite infection in mosquitoes (Hughes et al., 2012; Murdock et al., 2015). Overall, there are still many problems that need to be overcome when using *Wolbachia* infections to block the spread of malaria.

6.2 Intestinal Flora

During the development process of malaria parasites in mosquitoes, in addition to genetic variation, the intestinal microbiota also makes an integral contribution to the susceptibility of mosquitoes to infection. After the malaria parasite enters the mosquito body, it makes its way to and settles in the midgut; thus, there must be numerous complex interactions between the intestinal microbial colony and the malaria parasite. In fact, there is already evidence showing a strong connection. Previous opinions were that malaria fever prevented bacterial infections by halting the synthesis of

lipopolysaccharides, thereby interfering with the growth of bacteria, but the actual situation suggests that the coexistence of malaria and bacterial infections, such as gram-positive *Staphylococcus aureus* and gram-negative *Escherichia coli* infections, is common in the tropics where malaria is endemic. The intestinal microbiota has also been found to comprise a higher quantity of *Bifidobacterium* and *Streptococcus* species in healthy individuals than in *P. falciparum*-infected patients (Sahar Traoré et al., 2015). In addition, some microorganisms have also been shown to significantly affect the ability of mosquitoes to act as vectors by regulating the immune response in the mosquito body (Gabrieli et al., 2021). These results indicate that it would be beneficial to develop malaria transmission-blocking strategies, and even some vaccines, based on the intestinal flora.

However, studies on how the intestinal flora affects malaria parasites in mosquitoes are relatively rare. Most research has focused on the relationship between the clinical symptoms of malaria and the intestinal flora (Ashour and Othman, 2020). Despite this, the studies conducted so far have provided a good reference and guide for future in-depth studies. The co-feeding of *E. coli*, *S. aureus*, and *P. falciparum* gametocytes has been shown to significantly reduce the malaria parasites infection level in mosquitoes (Gabrieli et al., 2021) and secretions from some flora may play key roles in the specific mechanism of this inhibition. Romidepsin, a protein secreted by the intestinal flora, has been shown to limit *Plasmodium* infection in rodent animals (Saraiva et al., 2018). A strain of *Enterobacter* isolated from wild *Anopheles arabiensis* mosquito populations in Zambia has also been shown to interfere with the development of malaria parasites *via* the production of reactive oxygen species before they invade the midgut epithelial cells (Cirimotich et al., 2011). Such flora may also control *Plasmodium* infection by regulating the expression of some genes in the midgut of mosquitoes. A previous study indicated that the intestinal flora could increase the expression of TEP1 protein in the midgut of mosquitoes in a manner similar to RNAi silencing to promote *Plasmodium* infection, but the specific mechanism remains a mystery (Wang et al., 2013).

7 DISCUSSION

Although malaria prevention relies mainly on insecticide-treated bed nets and indoor residual spraying, major breakthroughs have been made in the prevention and control of malaria in the past decade. However, the number of malaria deaths worldwide has remained stable at around 400,000/year in recent years, which is a crucial fact that drives the development of global malaria prevention and control (Caragata et al., 2020). ACT and a variety of other preventive drugs, including primaquine, were seen as a new dawn in reducing global malaria deaths. However, the increasing number of drug-resistant strains has greatly impacted the effectiveness of these drugs and has become a great obstacle for local malaria prevention and control measures (Wang et al., 2021). Although many studies aim to respond to the current urgent situation by developing new antimalarial drugs or modifying existing drugs, because of the long cycle of drug development and other problems, there is currently no new drug that can resolve this situation. In addition, for many countries or regions, it is not simply a case of reducing the incidence of malaria, as the total eradication of malaria is their end goal. Under these conditions, in addition to the use of antimalarial drugs, it would be beneficial to find other more promising ways to block the spread of malaria in the natural environment, thus eliminating the possibility of malaria infection.

Unlike clinical malaria, the reproductive period of the malaria parasite in mosquitoes is key to its widespread transmission. An obvious benefit is that, if the propagation of malaria parasites in mosquitoes is blocked, not only can the infection of the population be reduced, but more importantly, it can also prevent the spread of existing drug-resistant strains, thereby prolonging the effectiveness and use of antimalarial drugs. After the entry of the malaria parasite gametocytes into the mosquito, they undergo the following sequential steps: leaving the host red blood cell, the conversion of the male and female gametocytes, fertilization, zygote invasion of the mosquito's midgut epithelium, and the subsequent oocyst development stages. Numerous studies have shown that several proteins from the different stages can be utilized as candidate targets for TBVs; however, there are still many difficulties to be overcome. For gametocyte surface proteins, only a small number of candidate proteins have been identified. What needs to be considered is how to produce these recombinant antibodies correctly and efficiently. Also in the future, the number of candidate target proteins can be increased through detection in areas of low-prevalence (Paul et al., 2016). In current research focusing on fertilization-related processes, whether interfering with the conversion of gametocytes to male and female gametocytes or with the combination of male and female gametocytes, excellent results have been achieved. However, the lack of innate immunogenicity of these proteins is an unavoidable problem, and a highly effective adjuvant without adverse side effects is essential for their practical use. Some scholars have suggested that, under the pressure of TBV, the ability of *Plasmodium* to increase male gametes to ensure transmission may be a test for the effectiveness of vaccines targeting male gametes (Teboh-Ewungkem et al., 2021). Further research is needed to clarify the specific mechanism for this. The targets in the post midgut developmental stage are different from those in the first two stages. The candidate proteins used can either be from the malaria parasite or from the mosquito's midgut. Therefore, a study attempted to genetically modify mosquitoes to block the development of malaria parasite oocysts in the midgut. Although there was a significant reduction in the development of the oocysts of the malaria parasite in the mosquito, it also caused a significant physiological burden on the mosquito itself (Dong et al., 2018). This suggests that in the direct genetic modification of mosquitoes, we need to be mindful of the impact on the physiological characteristics of the mosquito itself and their impact on the ecosystem. Moreover, the relevant antibody will only provide a transmission-blocking effect in the mosquito

when the mosquito sucks the blood of a recipient vaccinated with the TBV. Thus, some scholars have explored the possibility of a vaccine that either has a transmission-blocking ability or a benefit for clinical malaria during the period that it exists in the blood of the recipients. A whole-killed blood-stage vaccine may be worth exploring, as it not only provides protection against the blood-stage challenge, but also significantly inhibits the development of malaria parasites in mosquitoes with the help of parasite-specific IgG and the inflammatory cytokine MCP-1 (Zhu et al., 2016). Furthermore, there are two issues that need to be addressed. A multi-target vaccine for the reproductive phase did not achieve synergistic effects in previous experiments (Zheng et al., 2016). Researchers believe that it may interfere with the specificity of the antigen response, but the specific mechanism remains unclear. In addition, during the development of malaria parasite vaccines, the correct folding and modification of the protein should be studied, as this is crucial for determining whether it can correctly cause a vaccine response (Patra et al., 2015).

The transmission and infection of *Plasmodium* is not only a two-way effect between the host and the parasite but also involves many different microorganisms which play an important role. There is increasing evidence that these microorganisms could help significantly block the spread of *Plasmodium* parasites. *Wolbachia*, an endosymbiont that is widespread as a parasite in mosquitoes, has been shown to play an important role in blocking malaria transmission (Ross and Hoffmann, 2021). Owing to its reproductive characteristics during its parasite phase, the release of male mosquitoes infected with *Wolbachia* can directly kill the mosquito population in the target area. However, it is difficult to ensure that female mosquitoes are not also released. This presents a need for more stringent requirements in male and female separation technology (Caragata et al., 2020). *Wolbachia* itself can also inhibit the growth and development of *Plasmodium* in mosquitoes, although the specific mechanism is still unclear. In addition, the intestinal flora seems to be a potential target for blocking the transmission of malaria, but there are currently only a few relevant studies. The advantage of applying these biological control strategies is that they are not as complicated as vaccine development, and the technical requirements are lower. This suggests that biological control strategies may be practically applied as malaria control measures in the short term to address the current situation. Even so, we still need to consider the possible impact of these newly introduced organisms on the local ecology, devise possible response plans, and provide possible new alternative control strategies (Bellini et al., 2020).

What needs to be carefully considered if TBVs are used to block the transmission of malaria, is the frequency of vaccination, which should be determined to ensure that the antibodies can be maintained at an effective level. This requires numerous studies to determine the effective concentrations of the relevant antibodies. Meanwhile, there is currently no relevant research exploring whether TBVs have side effects in the human body. These are problems that should be resolved in the future. In addition, if *Wolbachia* is used to kill mosquitoes to stop the spread of malaria, the effects on the local ecosystem must be clarified. In general, although there is still a long way to go before their practical use, the development of TBVs and biological control strategies based on endosymbionts could significantly inhibit the widespread distribution of malaria. These directions show promise for alleviating the current malaria situation and assisting the end goal of malaria eradication.

AUTHOR CONTRIBUTIONS

YW and SY conceived and designed the study. SY wrote the draft of the manuscript. JW, XL, HZ, LW, XY, and YW improved all the versions. All authors contributed to the article and approved the submitted version.

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Anti-Gametocyte Antigen Humoral Immunity and Gametocytemia During Treatment of Uncomplicated Falciparum Malaria: A Multi-National Study

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Introduction: Understanding the human immune response to *Plasmodium falciparum* gametocytes and its association with gametocytemia is essential for understanding the transmission of malaria as well as progressing transmission blocking vaccine candidates.

Methods: In a multi-national clinical efficacy trial of artemisinin therapies (13 sites of varying transmission over South-East Asia and Africa), we measured Immunoglobulin G (IgG) responses to recombinant *P. falciparum* gametocyte antigens expressed on the gametocyte plasma membrane and leading transmission blocking vaccine candidates *Pfs*230 (*Pfs*230c and *Pfs*230D1M) and *Pfs*48/45 at enrolment in 1,114 participants with

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clinical falciparum malaria. Mixed effects linear and logistic regression were used to determine the association between gametocyte measures (gametocytemia and gametocyte density) and antibody outcomes at enrolment.

Results: Microscopy detectable gametocytemia was observed in 11% (127/1,114) of participants at enrolment, and an additional 9% (95/1,114) over the follow-up period (up to day 42) (total 20% of participants [222/1,114]). IgG levels in response to *Pf*s230c, *Pf*s48/45 and *Pf*s230D1M varied across study sites at enrolment ($p < 0.001$), as did IgG seroprevalence for anti-*Pf*s230c and D1M IgG ($p < 0.001$), but not for anti-*Pf*s48/45 IgG ($p = 0.159$). In adjusted analyses, microscopy detectable gametocytemia at enrolment was associated with an increase in the odds of IgG seropositivity to the three gametocyte antigens (*Pf*s230c OR [95% CI], *p*: 1.70 [1.10, 2.62], *0.017*; *Pf*s48/45: 1.45 [0.85, 2.46], *0.174*; *Pf*s230D1M: 1.70 [1.03, 2.80], *0.037*), as was higher gametocyte density at enrolment (per two-fold change in gametocyte density *Pf*s230c OR [95% CI], *p*: 1.09 [1.02, 1.17], *0.008*; *Pf*s48/45: 1.05 [0.98, 1.13], *0.185*; *Pf*s230D1M: 1.07 [0.99, 1.14], *0.071*).

Conclusion: *Pf*s230 and *Pf*s48/45 antibodies are naturally immunogenic targets associated with patent gametocytemia and increasing gametocyte density across multiple malaria endemic settings, including regions with emerging artemisinin-resistant *P. falciparum*.

Keywords: malaria, gametocyte, antibodies, falciparum malaria, clinical malaria, epidemiogy, immunity

INTRODUCTION

Malaria control currently relies on prompt diagnosis and effective first-line antimalarial treatment with Artemisinin Combination Therapies (ACTs) and the use of insecticide treated bed nets. However, resistance to the artemisinin derivatives in *Plasmodium falciparum* is now firmly established within the Greater Mekong Sub-Region (GMS) of South-East Asia (Ashley et al., 2014; Imwong et al., 2020) and emerging in other regions (Uwimana et al., 2021), threating the management and control of malaria. To prevent the spread of artemisinin resistant *P. falciparum*, GMS countries have committed to the elimination of all species of human malaria by the year 2030 (WHO, 2016). The future development of vaccines that have reduce transmission, known as transmission-blocking vaccines, are regarded as a priority to achieve malaria elimination goals globally (Beeson et al., 2019).

Elimination of *P. falciparum*, including artemisinin resistant parasites, is dependent on preventing the transmission of sexual stage parasites (gametocytes) between human and mosquito. Patent gametocytemia (microscopy detectable) has been observed more frequently in clinical malaria patients with slow-clearing *P. falciparum* infections characteristic of artemisinin resistance (Ashley et al., 2014), which may enhance the spread of these strains in regions where artemisinin resistance has emerged. The success of malaria transmission may depend on several factors including the acquisition of human immunity to key gametocyte antigens. Naturally acquired immunity, which develops after repeated exposure to *Plasmodium* spp., has been shown to protect against clinical disease and high densities of blood-stage parasites. Antibodies specific for *P. falciparum* gametocytes also develop with age and repeated exposure, and their ability to prevent transmission is realised in the mosquito where they have been demonstrated to reduce fertilisation and further development of transmissible forms of the parasite (Bousema et al., 2006; Drakeley et al., 2006; Stone et al., 2016; Stone et al., 2018). Thus, the presence of anti-gametocyte antibodies may infer gametocytemia as well as of the onward transmission potential of *P. falciparum* parasites, including drug resistant parasites.

Gametocytes of *P. falciparum* undergo a complex process of development marked by several morphological stages that occur within infected erythrocytes in the human host. The early gametocyte-infected erythrocyte stages sequester in the bone marrow and the spleen, and evidence for development of antibodies to these stages is conflicted (Chan et al., 2018; Dantzler et al., 2019). Few responses to mature gametocyte-infected erythrocytes have been studied in detail, however, studies have demonstrated the acquisition of human immunity to key antigens present on the surface of mature gametocytes (van Dijk et al., 2001; Eksi et al., 2006). Antibodies do occur to antigens on the surface of the gametocyte-infected erythrocyte, but they appear to be much less prominent and occur at low levels (Chan et al., 2018; Dantzler et al., 2019). *Pf*s230 and *Pf*s48/45 are the most well characterised antibody targets of *P. falciparum* gametocytes, with *Pf*s230 currently in Phase II trials (Healy et al., 2021). They are essential for fertilisation in the mosquito midgut, and meta-analyses of six studies evaluating the transmission blocking role of anti-*Pf*s230 and *Pf*s48/45 antibodies (Drakeley et al., 2004; Bousema et al., 2006; Drakeley et al., 2006; van der Kolk et al., 2006;

Bousema et al., 2007; Bousema et al., 2010) demonstrated that in ~57 - 69% of individuals these antibodies were shown to significantly reduce mosquito infection rates by >90% in standard membrane feeding assays (Stone et al., 2016). However, it is currently unknown how antibodies specific for gametocyte antigens vary within- and between different malaria endemic settings with varying gametocyte metrics. Here, we investigated the relationship between gametocytemia (prevalence and density at enrolment) and acquired anti- *Pfs*230 and *Pfs*48/45 antibody responses measured at enrolment in patients from South-East Asia and Africa participating a multinational trial of artemisinin treatment efficacy with varying rates of gametocytemia and artemisinin drug resistance.

METHODS

Study Design and Procedures

Plasma samples were acquired from 1,114 participants from 11 South-East Asian study sites in Thailand (three sites: Mae Sot, Srisaket, Ranong), Cambodia (four sites: Pailin, Preah Vihear, Ratanakiri, Pursat), Bangladesh (Ramu), Myanmar (Shwe Kyin), Lao PDR (Attapeu) and Vietnam (Binh Phuoc), and two African sites; Democratic Republic of Congo (Kinshasa) and Nigeria (Ilorin), participating in a multicentre open label randomised trial of artemisinin mono- and combination treatment efficacy, described previously (Ashley et al., 2014). Participants were eligible for inclusion if they presented with uncomplicated falciparum malaria and were randomised to receive a 3-day course of either 2mg/kg or 4mg/kg artesunate monotherapy, followed by a full 3-day course of ACT. Peripheral asexual and gametocyte density was measured by microscopy at enrolment and 4, 6, 8, and 12 hours, then every 6 hours following treatment until two consecutive parasite negative blood slides were observed. Parasite counts were used to determine the primary outcome of the study, parasite clearance half-life (hours), derived using the WWARN parasite clearance estimator (Flegg et al., 2011). Follow up assessments were performed on days seven, 14, 28 and 42. Genotyping of recurrent or newly acquired infections and the *kelch*13 marker were performed as previously described (Ashley et al., 2014). Informed consent was obtained from all participants/legal guardians, and ethical approval was granted by national ethics committees in each participating country, the Oxford Tropical Research Ethics Committee (06/11) and Alfred Hospital Committee for Ethics, Australia (485/12).

Recombinant Proteins

A truncated recombinant version of *Pfs*230, termed *Pfs*230D1M, based on the 3D7 allele was expressed in the mammalian HEK293 cell expression system as previously described (Chan et al., 2018). *Pfs*230D1M contains the first 6 cysteine domain of full-length protein (MacDonald et al., 2016), while *Pfs*230c contains the first 3 domains of full-length protein and expressed in a wheat germ cell-free expression system (Miura et al., 2013). Recombinant *Pfs*48/45 was produced in *L. lactis* and is a truncated form of full-length protein (Singh et al., 2015).

Antibody Determination

Plasma samples acquired at enrolment were used to determine levels of IgG to recombinantly expressed gametocyte antigens by high-throughput ELISA (JANUS liquid handling system, Perkin Elmer) as previously described (Ataide et al., 2017). Briefly, Spectraplates were coated with recombinant *P. falciparum* gametocyte stage antigens *Pfs*230c, *Pfs*230D1M and *Pfs*48/45 (0.5µg/mL) and incubated overnight. Plates were blocked for 2 hours at room temperature with 1% casein in 1 x PBS, and then incubated with patient and control sera at dilutions optimised separately for each antigen construct (1:250 for *Pfs*230c and 1:800 for *Pfs*230D1M and *Pfs*48/45) in 0.1% casein PBS at room temperature for 2 hours. Final serum concentrations were selected based on sample reactivity and avoidance of high end optical density saturation following titration of a random subset of samples (n=39) for each antigen, separately (**Supplementary Figure 1**). Goat anti-human Horse Radish Peroxidase (HRP)-conjugated IgG antibody was added at a concentration of 1/2500 diluted in 0.1% casein in 1 x PBS for one hour at room temperature. Between each incubation and addition step, plates were washed three times with 1 x PBS + 0.05% Tween20 using an automated plate washer. ABTS substrate was added to each well and covered for 30 minutes at room temperature, then stopped with 1% SDS, and read in a spectrophotometer at 405nm. For each plate, a total of six wells were incubated with positive control sera consisting of pooled samples from highly reactive naturally exposed donors from Papua New Guinea. Additionally, on each plate a panel of negative control sera collected from unexposed Melbourne donors were incubated in six wells per plate. Wells containing no test sera were used to deduct background reactivity from each sample. A seropositivity cut-off point was set at an OD above the mean + 2SDs of a panel of Melbourne donors.

Statistical Analyses

The distribution of the demographic, parasitological and antibody variables were described using median (25^{th}-75^{th} percentiles, range) or frequency (95% Confidence Interval [CI]) where appropriate. Mann-Whitney tests were performed to compare medians, associations between categorical variables were assessed using chi-squared tests, and correlations between antibody responses were estimated by calculating Spearman's Rho. Mixed effects linear and logistic regression was used to determine the effect of *kelch*13 on gametocyte outcomes (gametocytemia at enrolment and gametocytemia after treatment), including a random effect for study site. Mixed effects logistic regression was used to determine the effect of categorical (gametocyte smear positive/negative) and continuous (log_2 gametocyte density [/µL]) gametocyte measures at enrolment on the odds of IgG seropositivity measured at enrolment, and mixed-effects linear regression was used for continuous antibody outcomes (IgG level [log_e optical density]) measured at enrolment. In addition, the effect of known risk factors for gametocytemia anaemia (hematocrit [%] at enrolment) and duration of symptoms [self-reported days of fever prior to enrolment]) on antibody outcomes was measured. All models assessing antibody measures as the

outcome were adjusted for the potential confounder, age (years) and included a random effect for study site. Effect modification by the artemisinin resistance associated *kelch*13 genotype was assessed by comparing models with and without an interaction term between the *kelch*13 genotype and gametocytemia at enrolment using the likelihood ratio test. All analyses were performed using Stata version 15.

RESULTS

Participant Characteristics and Gametocytemia

IgG antibodies were measured at enrolment in 1,114 patients participating in a clinical efficacy study of artemisinin derivatives across 13 study sites in South East Asia and Africa. Reflecting broad differences in malaria epidemiology between sites, participants recruited from South-East Asian sites were predominantly male adults (78.2%, [770/985]); median age 24 years [25th–75th percentile: 18-34]), whereas participants recruited from African sites in Nigeria and the DRC, were all children (55% male (71/130); median age 4.5 years [25th–75th:3-6], **Table 1**). Microscopy detectable gametocytes were found in 11.4% (127/1,114) participants and median gametocyte density was 48 [16 – 192] gametocytes/μL at enrolment in gametocyte positive participants (**Table 2**). An additional 95 participants developed gametocytemia detected by microscopy in follow-up timepoints up until day 42, resulting in a total of 19.9% (222/1,114) of participants having any microscopy detectable gametocytemia throughout the study. The prevalence of gametocytemia at enrolment varied by study site, and was highest in Kinshasa, DRC (31.0% [37/118]) followed by Western Cambodian sites Pursat and Pailin (18.3% [22/120] and 19.2% [19/99], respectively) (**Table 2** and **Figure 1**), where artemisinin resistance is established and the proportion of participants infected with a *kelch*13 mutant strain was greatest (**Supplementary Table 1**) (Ashley et al., 2014). The odds of

TABLE 1 | Participant characteristics at each study site.

Country	Study Site	N	Age (years), Median (25th-75th percentiles, min-max)	% Male (n/N)
Africa				
Nigeria	Ilorin	11	4 (2-6, 0.7-8)	73 (8/11)
DRC	Kinshasa	119	5 (3-6, 0.7-8)	53 (63/119)
South-East Asia				
Bangladesh	Ramu	49	26 (20–35, 10-55)	86 (42/49)
Cambodia	Pursat	120	25 (19–33, 3-60)	91 (109/120)
	Preah Vihear	120	20 (14–29, 4-58)	68 (82/120)
	Ratanakiri	120	14 (9-19.5, 2-55)	65 (78/120)
	Pailin	99	25 (19–38, 10-57)	87 (86/99)
Laos	Attapeu	93	23 (14–29, 6-60)	69 (64/93)
Myanmar	Shwe Kyin	79	24 (19-31, 1-54)	82 (65/79)
Thailand	Mae Sot	120	29 (22.5–37, 18-58)	78 (94/120)
	Srisaket	41	29 (22–38, 16-54)	100 (41/41)
	Ranong	23	33 (19-53)	70 (16/23)
Vietnam	Binh Phuoc	120	26 (18.5–38.5, 3-61)	77 (92/120)

TABLE 2 | Gametocyte characteristics at each study site.

Country	Study Site	N	Gametocytemia at enrolment % (n/N)	Gametocyte density at enrolment[a], Median (25th-75th percentiles, min-max)	Gametocytemia during study period % (n/N)	Duration of gametocytemia[a] (hours), Median (25th-75th percentiles, min-max)
Africa						
Nigeria	Ilorin	11	0 (0/10)	–	0 (0/10)	–
DRC	Kinshasa	119	31 (37/118)	32 (16-80, 16-880)	46 (55/118)	72 (54-164, 2-335)
South-East Asia						
Bangladesh	Ramu	49	0 (0/49)	–	8 (4/49)	60 [b]
Cambodia	Pursat	120	18 (22/120)	80 (16-496, 16-12058)	25 (30/120)	168 (84-330, 18-344)
	Preah Vihear	120	5 (6/120)	56 (32-176, 32-304)	8 (10/120)	156 (72-333, 42-357)
	Ratanakiri	120	6 (7/120)	64 (32-144, 16-9294)	8 (10/120)	168 (138-168, 48-311)
	Pailin	99	19 (19/99)	32 (16-176, 16-1200)	27 (27/99)	120 (66-308, 8-332)
Laos	Attapeu	93	6 (6/93)	136 (16-304, 16-336)	8 (7/93)	131 (73-144, 50-162)
Myanmar	Shwe Kyin	79	11 (9/79)	4019 (32-4898, 16-20010)	24 (19/79)	126 (56-168, 18-334)
Thailand	Mae Sot	120	10 (12/119)	64 (24-216, 16-3552)	27 (32/120)	116 (54-155, 6-335)
	Srisaket	41	2 (1/40)	384 (-, -)[b]	12 (5/41)	20 (12-56, 12-84)
	Ranong	23	4 (1/23)	48 (-, -)[b]	26 (6/23)	122 (108-161, 36-332)
Vietnam	Binh Phuoc	120	6 (7/120)	144 (48-576, 32-1296)	11 (13/120)	168 (96-337, 38-354)

[a]*In gametocyte positive participants only.*
[b]*Only a single gametocyte positive participant.*

microscopy detectable gametocytemia was higher in participants infected with a *kelch*13 mutant *P. falciparum* strain compared to participants with a wild-type infection following treatment (Odds Ratio (OR) [95% Confidence Interval (CI)], *p value*: 2.39 [1.42, 4.03], *0.001*) but not at enrolment (OR [95% CI], *p value*: 0.86 [0.51, 1.45], *0.576*), nor was it associated with gametocyte density at enrolment or duration of microscopy detectable gametocytemia after treatment (**Supplementary Table 2**).

Between-Population Heterogeneity in Anti-Gametocyte Antigen IgG Responses

Total IgG levels and seroprevalence were determined at enrolment in response to the *P. falciparum* gametocyte antigens *Pfs*230c, *Pfs*230D1M and *Pfs*48/45. Overall, 65.8%, 15.7% and 11.9% of participants were categorised as seropositive for anti-*Pfs*230c anti-*Pfs*230D1M and anti-*Pfs*48/45 IgG, respectively. IgG seroprevalence varied between study sites for *Pfs*230c (range 44.5% - 82.6%, chi-squared test p *<0.001*) and *Pfs*230D1M (range 5.9% - 30.4%, chi-squared test p *<0.001*) (**Figure 1**). Comparatively, seroprevalence was lower for anti-*Pfs*48/45 IgG and did not vary significantly across sites (range 5.9% - 18.4%, chi-squared test p = *0.159*) (**Figure 1**). Overall, there was a trend toward lower seroprevalence in Nigeria and DRC (range: 6% – 45%) compared to Asian study sites (range: 7% – 82%, **Figure 1**). Total IgG levels specific for all three antigens varied across study sites (Kruksall-Wallis p *<0.001*, **Supplementary Figure 2**). Total IgG levels in response to gametocyte antigens and previously published asexual stage antigens were moderately but significantly correlated (Rho range 0.1372 – 0.5395 all p *<0.05*, **Supplementary Table 3**) (Ataide et al., 2017).

Quantifying the Association Between Gametocytemia and Anti-Gametocyte Antigen IgG

Seroprevalence of anti-gametocyte antigen IgG was greater in participants with microscopy detectable gametocytemia compared to gametocyte negative participants at enrolment in the majority of study sites (**Supplementary Figure 3**). In mixed-effects logistic regression (adjusted for age (years) and including a random effect for study site) microscopy detectable gametocytemia at enrolment was associated with a 45-70% increase in the odds of anti-gametocyte antigen IgG seropositivity (*Pfs*230c (OR) [95% (CI)], *p value*: 1.70 [1.10, 2.62], *0.017*; *Pfs*48/45: 1.45 [0.85, 2.46], *0.174*; *Pfs*230D1M: 1.70 [1.03, 2.80], *0.037*, **Table 3**). Additionally, a two-fold increase in gametocyte density at enrolment was associated with increases (5-9%) in the odds of IgG seropositivity (OR [95% CI], *p*, *Pfs*230c: 1.09 [1.02, 1.17], *0.008*; *Pfs*48/45: 1.05 [0.98, 1.13], *0.185*; *Pfs*230D1M: 1.07 [0.99, 1.14], *0.071*, **Table 3**). Similar associations between gametocytemia and levels of anti-

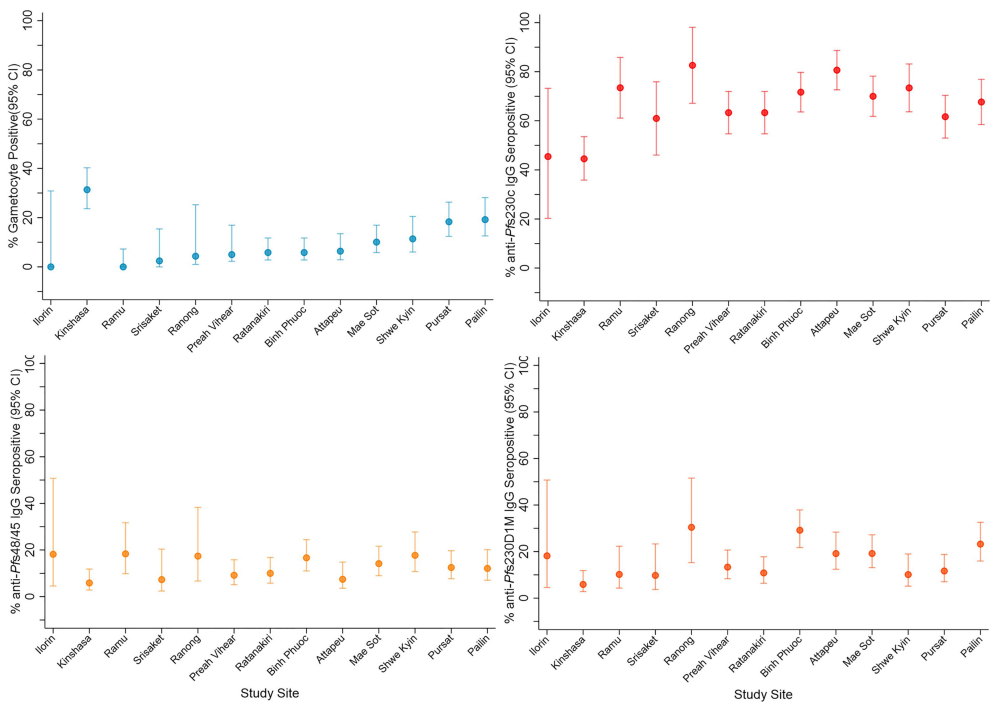

FIGURE 1 | Gametocyte and IgG prevalence (95%CI) in response to gametocyte targets. IgG seroprevalence varied across study sites for *Pfs*230cand *Pfs*230D 1M (chi-squared test p <0.001) but not *Pfs*48/45 (chi-squared test p = 0.159). Study sites are arranged by continent (Africa - Nigeria (Ilorin n=11), Democratic Republic of Congo (Kinshasa n=119); Asia - Laos PDR (Attapeu n=93), Bangladesh (Ramu n=49), Thailand (Mae Sot n=120, Srisaket n=41, Ranong n=23), Cambodia (Pailin n=99, Preah Vihear n=120, Ratanakiri n=120, Pursat n=120), Myanmar (Shwe Kyin n=79), and Vietnam (Binh Phuoc n=120) and then in order of lowest to highest prevalence of gametocytemia at enrolment.

TABLE 3 | Effect of gametocytemia and gametocyte density on IgG seroprevalence at enrolment.

	OR (95% CI), *p*		
	***Pfs*230c**	***Pfs*48/45**	***Pfs*230D1M**
Gametocytemia at enrolment[a]	1.70 (1.10, 2.62), *0.017*	1.45 (0.85, 2.46), *0.174*	1.70 (1.03, 2.80), *0.037*
Gametocyte density at enrolment[b]	1.09 (1.02, 1.17), *0.008*	1.05 (0.98, 1.13), *0.185*	1.07 (0.99, 1.14), *0.071*

OR – odds ratio, CI – confidence interval.

Estimates derived from mixed effects logistic regression adjusted for age (years) and specify a random effect for study site. [a]Estimate for participants gametocyte positive compared to gametocyte negative patients at enrolment [b]Estimate for a two-fold increase in gametocyte density (/μL).

gametocyte antigen IgG were also found (**Supplementary Table 4**). In addition, we also examined the association between hematocrit and duration of fever prior to enrolment and antibody outcomes, as these clinical variables are known to be associated with patent gametocytemia during uncomplicated *P. falciparum* malaria. Increasing hematocrit (%) was associated with a decrease in the odds of IgG seropositivity and a reduction in IgG level, and longer self-reported duration of fever prior to enrolment (days) was associated with an increase in the odds of IgG seropositivity and increased IgG levels (**Supplementary Table 5**), similar to their known associations with risk of patent gametocytemia (**Supplementary Table 6**).

To gain a greater understanding of the associations between gametocytemia and antibodies in the context of artemisinin resistant *P. falciparum* infections, we investigated whether these associations differed for participants infected with a wild-type or *kelch13* mutant *P. falciparum* strain. While the magnitude of association between microscopy detectable gametocytemia at enrolment and an increased odds of IgG seroprevalence was larger in participants infected with a *kelch13* mutant strain of *P. falciparum* (OR range 2.16-2.94) compared to those infected with a *kelch13* wild type strain (OR range 1.17-1.36), there was no evidence for effect modification by *kelch*13 genotype [*p* for interaction terms range *0.12 - 0.27* (**Table 4**)].

DISCUSSION

Antibodies specific for gametocyte stages may play a significant role in reducing the transmission of malaria. However, knowledge of the acquisition and prevalence of antibodies and how they relate to gametocytemia, and potential transmission of drug resistant *P. falciparum* is limited. We determined IgG levels and seroprevalence to the sexual stage *P. falciparum* antigens *Pfs*230 and *Pfs*48/45 in a multinational clinical efficacy trial of artemisinin therapy across study sites in South-East Asia and Africa, including regions of confirmed artemisinin resistance. Overall, anti-gametocyte antigen IgG responses varied significantly within and between study sites and were associated with microscopy detectable gametocytemia at enrolment and increased gametocyte density. Anti-gametocyte antigen antibodies may serve as markers of gametocyte exposure in malaria endemic populations, rather than biomarkers of active gametocytemia in individuals, and between study site variation suggests that the transmission potential of *P. falciparum* parasites, including drug resistant parasites, may vary between populations.

Identifying immunogenic gametocyte antigens is important for development of transmission blocking vaccines and could inform future development of serosurveillance tools. Seroprevalence of anti-*Pfs*230 IgG, but not *Pfs*48/45, varied by study site, which suggests that this antigen maybe more sensitive for delineating differences in gametocytemia and potential transmission blocking immunity across sites with varying transmission intensity. The observed reactivity and difference in seroprevalence across study sites may reflect differences in transmission intensity and prior exposure. Indeed, we have previously demonstrated significant variation in IgG responses specific for pre-erythrocytic and blood-stage parasite antigens in the same populations (Ataide et al., 2017), and anti-gametocyte antigen IgG was significantly correlated with these markers of transmission intensity and prior exposure in this study.

Anti-gametocyte antigen IgG seroprevalence was generally lower in participants recruited from DRC, a relatively higher transmission setting where patients were all children, but had the highest prevalence of gametocytes at enrolment, compared to

TABLE 4 | Gametocytemia and odds of IgG seroprevalence according to kelch13 genotype.

	OR (95% CI), *p*[a]		
	***Pfs*230c**	***Pfs*48/45**	***Pfs*230D1M**
Wild Type (n=743)	1.36 (0.81, 2.27), *0.24*	1.17 (0.57, 2.38), *0.67*	1.35 (0.70, 2.62), *0.37*
Mutant[b] (n=335)	2.94 (1.24, 6.98), *0.02*	2.16 (0.95, 4.93), *0.07*	2.68 (1.24, 5.79), *0.01*

p-value for likelihood ratio tests: Pfs230c = 0.12, Pfs48/45 = 0.27, Pfs230D1M = 0.18.

OR, odds ratio; CI, confidence interval.

Estimates derived from mixed effects logistic regression including an interaction term between gametocytemia and kelch13 genotype, adjusted for age (years) and a random effect specified for study site.

[a]Odds of IgG seropositivity in participants gametocyte positive compared to gametocyte negative participants.

[b]Single point mutations in the propeller domain of kelch13 after position 440.

participants recruited in Asian study sites, with relatively lower malaria transmission and where the majority of participants were adults. There may be several factors contributing to the lower seroprevalences observed in DRC which warrant further investigation. Firstly, despite the highest prevalence of gametocytemia at enrolment being observed in DRC, enrolment gametocyte density was generally lower compared to Asian study sites. Secondly, anti-gametocyte IgG responses have been shown to increase with age and exposure, as does their transmission reducing activity (Drakeley et al., 2006). Thirdly, geographical clustering of polymorphisms in gametocyte antigens has been demonstrated (Jones et al., 2015), which may differentially impact recognition of the gametocyte antigen constructs utilised across populations. Additionally, duration of gametocytemia, which may have differed between study sites, is likely to influence antibody acquisition and/or boosting, however, the duration of gametocytemia prior to clinical presentation and enrolment into the study is unknown. While the reasons for differential anti-gametocyte antigen IgG between DRC and Asian sites remain to be elucidated, our findings provide important data from Asian study sites and demonstrate that anti-gametocyte antigen responses are associated with patent circulating gametocytemia in low transmission settings. This is important given the paucity of studies assessing the prevalence of anti-gametocyte responses in low transmission settings when compared to studies in populations in moderate to high transmission settings (Stone et al., 2016).

Some patients had anti-gametocyte antigen antibodies in the absence of gametocytes, potentially a remnant from a previous infection or undetected gametocytes in the current infection. Microscopy misses low density gametocytemia and recent studies utilising molecular detection of sexual stages indicate that most infected individuals carry gametocytes (Schneider et al., 2007; Shekalaghe et al., 2007). The antibodies observed may be indicative of antibody boosting upon release of late-stage gametocytes at densities not detected by microscopy (i.e., at sub-microscopic densities). This hypothesis is supported by previous studies, which have observed rapid development of anti-gametocyte IgG responses following gametocyte exposure (Bousema et al., 2010; Skinner et al., 2015). Additionally, known risk factors of patent gametocytemia in clinical *P. falciparum* infection anaemia (estimated here using reduced hematocrit [%]), and increased duration of infection [duration of fever prior to enrolment (days)] (Price et al., 1999), which may act as proxy measures for gametocytemia during the current infection, were also associated with anti-gametocyte antigen antibodies. Finally, anti-asexual IgG responses were highly correlated with anti-gametocyte antigen responses in this study, which may further infer greater duration of infection in participants seropositive for and with greater levels of anti-gametocyte antigen IgG responses. Together, these findings further demonstrate that anti-gametocyte antigen IgG seropositivity is associated with patent gametocytemia and may act as a marker of exposure to gametocytemia within a population.

In this cohort, gametocytemia following treatment was higher in participants infected with a mutant *kelch*13 strain, and a systematic review has also demonstrated that artemisinin treatment failure is associated with a 15-fold greater risk of gametocytemia (WWARN, 2016). We observed greater odds of IgG seroprevalence with patent gametocytemia and increased gametocyte density at enrolment, however, we did not find statistically significant evidence of effect modification by *kelch*13 genotype on this association at enrolment despite biologically relevant differences in the odds of seroprevalence in genotype stratified analysis. Further investigation of this association in therapeutic efficacy trials following treatment are warranted to determine the impact of artemisinin resistance on gametocytemia and resulting antibody dynamics. While the transmission-blocking activity has been established for anti-*Pfs*230 and *Pfs*48/45 IgG antibodies (Stone et al., 2016; Stone et al., 2018), and residual infection following ACT treatment has been associated with longer gametocyte carriage and a subsequent greater risk of mosquito infection (Beshir et al., 2013), the ability of anti-gametocyte antigen antibodies to limit the onward transmission of artemisinin resistant *P. falciparum* across various transmission settings, however, is unknown and warrants further investigation.

A major strength of this study was that it included participants from diverse epidemiological settings across Africa and South-East Asia. The study enrolled patients with uncomplicated clinical malaria into a therapeutic efficacy study, and while they serve as sentinel surveillance populations for emerging drug resistance, the generalisability of our findings to individuals living in the community with asymptomatic malaria and gametocytemia is unknown. We included the two best characterised gametocyte antigens, which have demonstrated transmission blocking activity in animal models and by standard membrane feeding assay (Miura et al., 2013; Singh et al., 2015; MacDonald et al., 2016). However, the transmission blocking potential of antibodies measured in this clinical cohort were not directly quantified. Further, transmission blocking activity has been demonstrated in participant samples depleted of both anti-*Pfs*230 and *Pfs*48/45 specific IgG (Stone et al., 2018), implicating additional regions of the *Pfs*230 and *Pfs*48/45 proteins or further gametocyte targets in transmission blocking activity of human antibodies which warrant further investigation. Future studies of the relationship between gametocyte prevalence and dynamics of these and additional antibody responses longitudinally, as well transmission-blocking activity of these responses, particularly in asymptomatic populations, will further inform transmission potential of *P. falciparum*, including drug resistant parasites.

CONCLUSIONS

In a multinational therapeutic efficacy trial of artemisinins in clinical malaria patients, we found antibodies against gametocyte antigens *Pfs*230c, *Pfs*230D1M and *Pfs*48/45 were associated with patent gametocytemia across populations from varying malaria endemicity. These findings further our understanding of acquired antibody responses to gametocytes, advancing

deepening our understanding of antibody responses to progress transmission-blocking vaccine candidates.

DATA AVAILABILITY STATEMENT

The datasets presented in this article are not readily available because reasonable requests to the access of study data will be considered upon application to the corresponding authors. De-identified, individual participant data will be available to researchers whose proposed purpose of use is approved by the data access committee at Mahidol Oxford Tropical Medicine Research Unit. Inquiries or requests for the data may be sent to datasharing@tropmedres.ac. Requests to access the datasets should be directed to katherine.oflaherty@burnet.edu.au or freya.fowkes@burnet.edu.au.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Alfred Hospital Human Research and Ethics Committee, Australia, the Oxford Tropical Research Ethics Committee, United Kingdom, and relevant national ethics committees (Thailand: Ethics Committee of the Faculty of Tropical Medicine, Mahidol University and Tak Province Community Ethics Advisory Board (T-CAB), Cambodia: National Ethics Committee for Health Research, Ministry of Health, Kingdom of Cambodia Institutional Review Board and National Institute of Allergy and Infectious Diseases, Bethesda, Maryland, USA, Myanmar: The Government of the Republic of the Union of Myanmar, Ministry of Health, Department of Medical Research (Lower Myanmar), Laos: Ministry of Health. National Ethics Committee for Health Research, Lao Peoples' Democratic Republic, Nigeria: Ethical Review Committee, University of Ilorin Teaching Hospital, Ilorin, Nigeria, Bangladesh: National Research Ethics Committee, Bangladesh Medical Research Council, Democratic Republic of the Congo: Republique Democratique du Congo, Ministere de l'Enseignement Superieur, Universitaire et Recherche Scientifique, Universite de Kinshasa, Ecole de Sante Publique Comite d'Ethique and Viet Nam: Ethics Committee for biomedical research of the Ministry of Health, Institute of Malariology-Parasitology-Entomology, Ho Chi Minh City). Written informed consent to participate in this study was provided by all participants/participants legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

EA, FN, JS, NW, JB, and FF designed the research protocol. KO'F, J-AC, JC, SZ, AP, DD, AD, ND, MD, RF, PL, CA, SP, TH, YH, MM, MF, OM, MO, CF, FN, JB, JS, NW, and FF performed the research, J-AC, DD, ET, TT, MT, JB, and FF provided reagents/analytical tools. KO'F, JC, JS, and FF wrote the manuscript. All authors provided critical revision to the manuscript and have approved the final version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2022.804470/full#supplementary-material

Supplementary Figure 1 | Serum dilution optimisation for anti-*Pfs*230D1M, *Pfs*48/45 and *Pfs*230C IgG in a random sub-sample of participant samples (n = 39).

Supplementary Figure 2 | Gametocyte density at enrolment (log_e transformed median, 25th and 75th percentiles) and IgG level (log_2 transformed median, 25th and 75th percentiles) in response to gametocyte targets *Pfs*230c, *Pfs*48/45 and *Pfs*230D 1M. IgG level varied by study site (Kruksall-Wallis $p<0.001$). Study sites are arranged by continent (Africa - Nigeria (Ilorin n=11), Democratic Republic of Congo (Kinshasa n=119); Asia - Laos PDR (Attapeu n=93), Bangladesh (Ramu n=49), Thailand (Mae Sot n=120, Srisaket n=41, Ranong n=23), Cambodia (Pailin n=99, Preah Vihear n=120, Ratanakiri n=120, Pursat n=120), Myanmar (Shwe Kyin n=79), and Vietnam (Binh Phuoc n=120) and then in order of lowest to highest prevalence of gametocytemia at enrolment.

Supplementary Figure 3 | IgG seroprevalence (95% CI) in participants negative (red) and positive (orange) for gametocytes at enrolment. Study sites are arranged by continent (Africa - Nigeria (Ilorin n=11), Democratic Republic of Congo (Kinshasa n=119); Asia - Laos PDR (Attapeu n=93), Bangladesh (Ramu n=49), Thailand (Mae Sot n=120, Srisaket n=41, Ranong n=23), Cambodia (Pailin n=99, Preah Vihear n=120, Ratanakiri n=120, Pursat n=120), Myanmar (Shwe Kyin n=79), and Vietnam (Binh Phuoc n=120)) and then in order of lowest to highest prevalence of gametocytemia at enrolment.

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