

qRT-PCR versus IFA-based Quantification of Male and Female Gametocytes in Low-Density *Plasmodium falciparum* Infections and Their Relevance for Transmission

Maria Gruenberg,^{1,2} Natalie E. Hofmann,^{1,2} Elma Nate,³ Stephan Karl,^{3,a} Leanne J. Robinson,^{3,b} Kjerstin Lanke,⁴ Thomas A. Smith,^{1,2} Teun Bousema,^{4,c} and Ingrid Felger^{1,2,c}

¹Swiss Tropical and Public Health Institute and ²University of Basel, Basel, Switzerland; ³Papua New Guinea Institute of Medical Research, Madang, Papua New Guinea; and ⁴Radboud University Medical Center, Nijmegen, The Netherlands

Background. Accurate quantification of female and male gametocytes and sex ratios in asymptomatic low-density malaria infections are important for assessing their transmission potential. Gametocytes often escape detection even by molecular methods, therefore ultralow gametocyte densities were quantified in large blood volumes.

Methods. Female and male gametocytes were quantified in 161 PCR-positive *Plasmodium falciparum* infections from a cross-sectional survey in Papua New Guinea. Ten-fold concentrated RNA from 800 μ L blood was analyzed using female-specific *pfs25* and male-specific *pfmget* or *mssp* qRT-PCR. Gametocyte sex ratios from qRT-PCR were compared with those from immunofluorescence assays (IFA).

Results. Gametocytes were identified in 58% (93/161) *P. falciparum*-positive individuals. Mean gametocyte densities were frequently below 1 female and 1 male gametocyte/ μ L by qRT-PCR. The mean proportion of males was 0.39 (95% confidence interval, 0.33–0.44) by *pfs25/pfmget* qRT-PCR; this correlated well with IFA results (Pearson's $r^2 = 0.91$; $P < .001$). A Poisson model fitted to our data predicted 16% *P. falciparum*-positive individuals that are likely to transmit, assuming at least 1 female and 1 male gametocyte per 2.5 μ L mosquito bloodmeal.

Conclusions. Based on model estimates of female and male gametocytes per 2.5 μ L blood, *P. falciparum*-positive individuals detected exclusively by ultrasensitive diagnostics are negligible for human-to-mosquito transmission.

Keywords. male gametocytes; sex ratio; transmission reservoir; low density; *Plasmodium falciparum*; ultrasensitive diagnostics.

Asymptomatic, low-density *Plasmodium* infections, if untreated, may present a silent reservoir for ongoing malaria transmission. DNA extraction from large blood volumes in combination with highly sensitive molecular diagnostic methods detected a surprisingly large reservoir of ultralow *Plasmodium falciparum* infections in malaria-endemic areas [1–3]. To what extent such low-density infections can sustain malaria transmission is not clear, as scanty parasitemia diminishes the likelihood of

a mosquito bloodmeal harboring both male and female gametocytes, a prerequisite for infectiousness to mosquitoes. The density of gametocytes in the host's blood, the maturity of gametocytes, and proportion of male gametocytes are critical determinants for successful transmission to the mosquito [4, 5]. Mosquito feeding assays (MFA) are ideally used to assess the infectiousness of a parasitemic host to mosquitoes [6–9], but integrating MFAs into large epidemiological field studies is logistically and technically challenging. Therefore, gametocyte density is often used as a surrogate to describe the infectious reservoir in epidemiological studies [10].

Gametocyte densities in asymptomatic parasite carriers are commonly below the microscopic detection threshold. Therefore, more sensitive molecular assays are employed, which target transcripts specifically expressed in gametocytes. The most sensitive *P. falciparum* gametocyte marker is *pfs25*, which is expressed in female gametocytes. Densities below 10 female gametocytes/ μ L blood are commonly observed by quantitative reverse transcription polymerase chain reaction (qRT-PCR) or other molecular methods targeting *pfs25* in submicroscopic infections [11–14]. Using female-specific transcripts alone without

Received 9 April 2019; editorial decision 12 August 2019; accepted 14 August 2019; published online August 22, 2019.

Correspondence: I. Felger, PhD, Swiss Tropical and Public Health Institute, Socinstrasse 57, 4002 Basel, Switzerland (ingrid.felger@unibas.ch).

^aPresent affiliation: Australian Institute of Tropical Health and Medicine, James Cook University, Australia.

^bPresent affiliation: Burnet Institute of Medical Research, Melbourne, Australia.

The Journal of Infectious Diseases® 2020;221:598–607

© The Author(s) 2019. Published by Oxford University Press for the Infectious Diseases Society of America. This is an Open Access article distributed under the terms of the Creative Commons Attribution-NonCommercial-NoDerivs licence (<http://creativecommons.org/licenses/by-nc-nd/4.0/>), which permits non-commercial reproduction and distribution of the work, in any medium, provided the original work is not altered or transformed in any way, and that the work is properly cited. For commercial re-use, please contact journals.permissions@oup.com
DOI: 10.1093/infdis/jiz420

any male-specific molecular marker underestimates total gametocyte density [15]. Highly expressed male gametocyte markers were identified by a recent transcriptome analysis of sorted female and male gametocytes [16]. *pfmget* (PF3D7_1469900) and *mssp* (PF3D7_1311100) are among the most highly expressed male transcripts [16].

Simultaneous detection of female as well as male gametocyte transcripts would not only provide more accurate gametocyte quantification but also permit analysis of the ratio of female and male gametocytes in a peripheral blood sample. Such knowledge is crucial to better understand the potential infectiousness of low gametocyte densities. Sex ratios in natural *P. falciparum* infection are highly variable and can adapt in response to anemia, the host's immune response, coinfecting parasite clones, or gametocyte density [17–20]. A recent human mosquito infectivity model based on molecular quantification of female and male gametocytes suggested that infectivity of low-density infections might be primarily impeded by the lack of male gametocytes [21]. In that model, male gametocyte densities below 10 per μL resulted in a 50% decrease in the proportion of infected mosquitoes [21]. This emphasized the importance of quantifying female and male gametocytes, particularly in individuals with low-density *Plasmodium* infections. Gametocyte sex ratio is also important in characterizing the transmission-blocking effects of antimalarial drugs [20, 22].

The current study addressed the question of how many low-density malaria infections could potentially be infectious to mosquitoes based on the presence of at least 1 female and 1 male gametocyte in 2.5 μL blood, the estimated size of a mosquito bloodmeal. Such data would be highly relevant for the design of malaria control interventions. We hypothesized that both female and male gametocytes can be quantified by optimized methods even in community samples of very low parasitemia. We used 161 blood samples from asymptomatic study participants from Papua New Guinea that were detected as *P. falciparum*-positive by highly sensitive molecular methods [3]. Almost 50% of these *P. falciparum* infections contained ultralow parasite densities that remained undetected by standard qPCR [3]. For detection of female and male gametocytes in such low-density samples, we analyzed 10-fold concentrated RNA from 800 μL blood using 2 highly expressed male gametocyte markers, *pfmget* and *mssp*, together with the established female marker *pfs25*. To validate the accuracy of these qRT-PCR assays for low-density infections, we compared sex ratio estimates obtained from qRT-PCR and immunofluorescence assays (IFA) after gametocyte enrichment. Based on these data we estimated the probability of *P. falciparum* infections with 1 female and 1 male gametocyte per 2.5 μL blood, and explored the risk of low-density infections contributing to onward transmission to mosquitoes.

METHODS

Study Design and Sample Collection

A cross-sectional survey was conducted in 300 participants older than 5 years from 2 coastal villages in Madang Province, Papua New Guinea, during the rainy season between November 2016 and February 2017. The study area is characterized by moderate transmission [23]. Mean age among study participants was 30 years (SD, 16.6; median, 31 years [range 15–44]). Of the 300 participants, 161 were *P. falciparum*-positive by standard or ultrasensitive qPCR on fingerprick or large-volume blood samples; 46.6% (75/161) of *P. falciparum*-positive participants were coinfecting with *P. vivax*. The study design and demographics were described previously [3].

After health status assessment and written informed consent, 5 mL venous blood was collected into sodium heparin coated vacutainers (BD Biosciences). For gametocyte detection, an 800 μL aliquot was immediately transferred into 4 mL RNAProtect Cell Reagent (Qiagen). One mL venous blood was kept at 37°C until further processing for magnetic-assisted cell sorting (MACS) purification of gametocytes, performed at 37°C, on the same day.

Ethical Approval

The study received ethical approval from Papua New Guinea Institute of Medical Research Institutional Review Board (IRB number 1516) and the Medical Research Advisory Committee of the Papua New Guinea Ministry of Health (MRAC number 16.01).

Molecular Gametocyte Quantification by qRT-PCR

RNA was extracted from 161 *P. falciparum*-positive samples as described previously [3]. For gametocyte quantification the Luna Universal One-Step RT-qPCR Kit (New England Biolabs) was used in a final reaction volume of 14 μL with 714 nm forward and reverse primers, 250 nm probe, and 4 μL RNA. *pfs25* primer and probe sequences were described previously [24]. Probe-based *pfmget* and *mssp* qRT-PCR assays were designed in gene regions described previously [15, 25] (Supplementary Table 1). The thermal cycle profile was 10 minutes at 55°C and 1 minute at 95°C followed by 45 cycles of 10 seconds 95°C and 1 minute at 58°C. In order to maximize test sensitivity, 4 μL of RNA, corresponding to an equivalent of 40 μL blood, were used per reaction. Female and male gametocyte densities were quantified using a 10-fold serial dilution of DNase-digested RNA of sex-sorted gametocytes [14], equivalent to 1000–0.01 gametocytes/ μL . *pfs25* and *pfmget* qRT-PCR assays showed similar sensitivity (limit of detection was 0.03 female gametocytes/ μL [95% confidence interval, CI, 0.01–18.46 female gametocytes/ μL] for *pfs25* and 0.03 male gametocyte/ μL [95% CI, 0.01–13.63 male gametocytes/ μL] for *pfmget* qRT-PCR). Compared to the marker *pfmget*, *mssp* qRT-PCR was less sensitive. A cutoff for positivity was set to 1 gametocyte/800 μL (0.001 gametocyte/

μL). Details of gametocyte assays are provided in [Supplementary Figure 1](#) and [Supplementary Tables 2–5](#).

MACS Enrichment of Gametocytes From Field Samples for IFA

Magnetic enrichment of gametocytes was carried out following the manufacturer's instructions (Miltenyi Biotec) and modified as described previously [26]. To avoid exflagellation of male gametocytes, liquids (blood and buffers) and equipment (column and magnet) were constantly kept at 37°C during the enrichment procedure. Enriched gametocytes in phosphate-buffered saline were distributed as thick drops on 3 slides per sample and air dried. Each slide contained enriched gametocytes from approximately 100 μL whole blood corresponding to a 20- to 30-fold concentration. Slides were fixed with ice-cold methanol and stored at -20°C prior to immunofluorescence staining.

Immunofluorescent Assays

Both sexes of gametocytes were stained using 1:500 diluted mouse IgG1 mAb α -Pfs16 (kind gift from Robert Sauerwein). Female gametocytes were stained in a 1:1000 dilution of rabbit- α -Pfg377 serum (kind gift from Pietro Alano [27]). Secondary antibodies, Alexa Fluor 488-conjugated goat- α -mouse-IgG1 (Life Science Technology) and Alexa Fluor 568-conjugated goat- α -rabbit-IgG1 (Life Science Technology) were diluted 1:250. Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI). Images were taken at 1080-fold magnification using a Leica DM5000B microscope.

Data Analysis

Cycle threshold value (Ct) values obtained from qRT-PCR were converted into gametocyte densities based on serial dilutions of female and male sorted gametocytes (1000–0.01 gametocytes/ μL), adjusted according to the factor of RNA template concentration with respect to whole blood, and \log_{10} transformed. Total gametocyte densities were reported as the sum of female and male gametocytes/ μL blood. Throughout the manuscript, sex ratio was defined as the proportion of males, in line with previous publications [15]. Statistical significance between proportions was determined by a X^2 test. Differences in female and male gametocyte density and sex ratios in *P. falciparum*-positive individuals were compared by Mann-Whitney U test. The agreement between IFA- and qRT-PCR-derived sex ratios was analyzed by Bland-Altman. Uni- and multivariable logistic regression was used to analyze the effect of covariates on the odds of total gametocyte density or sex ratio. Statistical analysis was performed using R (version 3.5.0).

We assume a mosquito bloodmeal volume of 2.5 μL , with the number of gametocytes ingested following Poisson distributions (ie, assuming that the gametocytes are not clustered within the blood). Hence for each sample the probability that a bloodmeal contains at least 1 female gametocytes is estimated as $g_f = 1 - \exp(-2.5d_f)$, where d_f is the overall density

of female gametocytes; correspondingly, for male gametocytes $g_m = 1 - \exp(-2.5d_m)$ and the probability that both sexes are present is the product $g_f g_m$. Ignoring variation between hosts in the numbers of mosquitoes biting them, the proportion of mosquito bites that potentially result in infection of the vector is then estimated as the mean of $g_f g_m$ over all 300 surveyed hosts (with PCR-negative samples taking a value of zero).

RESULTS

Gametocyte Positivity by Sex-Specific Markers in Community Samples From Papua New Guinea

Using ultrasensitive detection methods, 161/300 study participants were *P. falciparum* positive [3]. By *pfs25* qRT-PCR 51% (82/161) of parasite carriers were gametocyte positive [3]. Because we introduced a positivity cutoff for gametocyte carriage of 1 gametocyte/800 μL sample (0.001 gametocyte/ μL), 9/93 of the earlier reported gametocytemic individuals [3] were considered negative and excluded from the present analysis. These earlier findings, based on *pfs25* qRT-PCR alone, were now complemented with 2 male gametocyte-specific qRT-PCR assays, *pfmget* and *mssp* (Table 1). These 3 *P. falciparum* gametocyte assays combined yielded a gametocyte positivity of 58% (93/161) among 161 *P. falciparum*-positive individuals. *pfs25* detected the greatest proportion of gametocyte carriers (88%; 82/93), followed by *pfmget* (81%; 75/93) and *mssp* (79%; 73/93). Only 65% (60/93) of all gametocyte-positive samples were detected simultaneously by all 3 markers. Markers *pfmget* and *mssp* differed in their detection of male gametocytes (Table 1). Of 93 individuals who were gametocyte positive by any of the 3 markers, 95% (88/93) carried male gametocytes by any male-specific marker. Among carriers of male gametocytes, 17% (15/88) were *pfmget* positive but *mssp* negative, while 15% (13/88) were *mssp* positive but *pfmget* negative. Although *pfmget*-qRT-PCR has a 4-fold improved sensitivity over *mssp*-qRT-PCR, more samples were exclusively detected by *mssp* (7/88) than exclusively by *pfmget* (4/88) (P value $< .01$).

Gametocyte Densities

Total gametocyte density by marker pair *pfs25/pfmget* in 86 gametocyte carriers was similar to that by *pfs25/mssp* in 89 gametocyte carriers (P value = .89) (Table 1). Geometric mean male density was 0.16 gametocytes/ μL (95% CI, 0.07–0.35) in 75 *pfmget*-positive samples and similar in 73 *mssp*-positive samples (P value $< .001$) (Table 1). Male gametocyte densities correlated well for both male-specific markers (Pearson $R^2 = 0.92$, P value $< .001$). Use of marker *pfs25* alone for gametocyte detection, resulted in lower densities and would underestimate total gametocyte density by 27% determined by Bland-Altman.

Sex-Specific Gametocyte Densities by *P. falciparum* Detection Methods

Gametocyte-positive samples were stratified according to whether *P. falciparum* infections were detected by 18SrRNA

Table 1. Gametocyte Densities and Sex Ratios in 161 Plasmodium falciparum-Positive Individuals from Papua New Guinea Stratified by Molecular Detection Methods for Asexual Parasites

Measures	st-qPCR ^a	st-qPCR– us-qPCR ^b	st-qPCR– us-qPCR– hv-us- qPCR ^c	Any Diagnostic Method
qPCR, ^d % (n/N)				
Asexual positivity	54.0 (87/161)	9.3 (15/161)	36.7 (59/161)	100.0 (161/161)
<i>P. vivax</i> coinfection	23.0 (37/161)	5.0 (8/161)	18.6 (30/161)	46.6 (75/161)
qRT-PCR				
Total gametocyte positivity, % (n/N)				
Any marker				
<i>pfs25</i> , <i>pfmget</i> , <i>mssp</i>	44.1 (71/161)	7.5 (12/161)	6.2 (10/161)	57.8 (93/161)
Marker pairs				
<i>pfs25</i> / <i>pfmget</i>	41.0 (66/161)	6.8 (11/161)	5.6 (9/161)	53.4 (86/161)
<i>pfs25</i> / <i>mssp</i>	43.5 (70/161)	7.4 (12/161)	4.3 (7/161)	55.3 (89/161)
Total gametocyte density, g/μL, geometric mean (95% CI)				
<i>pfs25</i> / <i>pfmget</i>	0.56 (0.23–1.37)	0.04 (0.01–0.10)	0.02 (0.01–0.04)	0.28 (0.13–0.58)
<i>pfs25</i> / <i>mssp</i>	0.52 (0.24–1.14)	0.06 (0.01–0.09)	0.02 (0.02–0.05)	0.28 (0.14–0.55)
Male gametocyte positivity, % (n/N)				
<i>pfmget</i>	36.0 (58/161)	5.6 (9/161)	5.0 (8/161)	46.6 (75/161)
<i>mssp</i>	38.5 (62/161)	5.0 (8/161)	1.9 (3/161)	45.3 (73/161)
Male gametocyte density, g/μL, geometric mean (95% CI)				
<i>pfmget</i>	0.35 (0.14–0.86)	0.02 (0–0.06)	0.01 (0–0.01)	0.16 (0.07–0.35)
<i>mssp</i>	0.24 (0.11–0.73)	0.02 (0.01–0.06)	0.01 (0–0.01)	0.16 (0.08–0.31)
Female gametocyte positivity, % (n/N) ^e				
<i>pfs25</i>	40.4 (65/161)	6.8 (11/161)	3.7 (6/161)	50.9 (82/161)
Female gametocyte density, g/μL, geometric mean (95% CI)				
<i>pfs25</i>	0.35 (0.15–0.82)	0.02 (0.01–0.06)	0.02 (0–0.03)	0.20 (0.1–0.40)
Proportion of males, ^f mean (95% CI)				
Marker pair <i>pfs25</i> / <i>pfmget</i>	0.37 (0.31–0.43)	0.43 (0.16–0.70)	0.32 (0.08–0.56)	0.39 (0.33–0.44)
Marker pair <i>pfs25</i> / <i>mssp</i>	0.40 (0.32–0.48)	0.49 (0.21–0.77)	0.29 (0–0.70)	0.41 (0.34–0.49)

Abbreviations: CI, confidence interval; hv, high volume; qRT-PCR, quantitative reverse transcription polymerase chain reaction; st, standard; us, ultrasensitive.

^a*P. falciparum*-infected individuals detected by standard qPCR (st-qPCR+).

^b*P. falciparum*-infected individuals additionally detected by ultrasensitive qPCR on fingerprick blood volume (us-qPCR).

^c*P. falciparum*-infected individuals additionally detected by ultrasensitive qPCR on high volume blood samples (hv-us qPCR).

^dData was previously published by Hofmann et al [3].

^eNine of 93 gametocyte carriers positive by *pfs25* qRT-PCR previously published by Hofmann et al [3] fell below positivity threshold of 0.001 gametocyte/μL blood and were excluded from our analysis.

^fProportion of males determined in samples >10 gametocytes (female + male)/800 μL blood.

qPCR in fingerprick samples (standard qPCR) or whether they were only detectable by ultrasensitive *varATS* qPCR (us-qPCR) or only in a large blood volume by ultrasensitive qPCR (hv-us-qPCR) [3]. Of 87 *P. falciparum*-positive samples by standard qPCR, 75% (65/87) carried female gametocytes by *pfs25* and 67% (58/87) or 71% (62/87) carried male gametocytes by *pfmget* or *mssp* qRT-PCR (Table 1). Geometric mean densities were similar by *pfs25* (0.35 female gametocytes/μL; 95% CI, 0.15–0.82) and by *pfmget* (0.35 male gametocytes/μL; 95% CI, 0.14–0.82; *P* value = .95) and slightly higher than by *mssp* (0.24 male gametocytes/μL; 95% CI, 0.11–0.73; *P* value = .60) (Figure 1A).

In 15 *P. falciparum* infections additionally detected by us-qPCR [3], 80% (12/15) carried gametocytes of either sex. In contrast, a significantly lower percentage of *P. falciparum* infections additionally detected by using hv-us-qPCR, only 16.9% (10/59) carried female and/or male gametocytes (*P* value < .001).

Geometric mean total gametocyte densities in *P. falciparum*-positive individuals detected by us-qPCR and hv-us qPCR were 6- and 11-fold lower (*P* value < .02 for us-qPCR; *P* value < .001 for hv-us-qPCR), compared to gametocyte densities in individuals detected by standard qPCR.

Validation of qRT-PCR-Based Sex Ratio Estimates by IFA

To complement the validation of molecular and IFA-derived sex ratio estimates in synchronized NF54 stage V gametocytes, thick smears from 51 gametocyte-enriched blood samples of natural *P. falciparum* infections were used for IFA (details are provided in Supplementary Figure 2). Gametocytes were detected in 23/51 smears (Figure 2A). A subset of samples (14/23 smears) with ≥10 gametocytes/slide (mean density 48.7 gametocytes/slide; 95% CI, 25.7–92.2) were available to compare sex ratios by qRT-PCR and IFA. The mean proportion of male gametocytes by IFA was 0.43 (95% CI, 0.31–0.53) and significantly

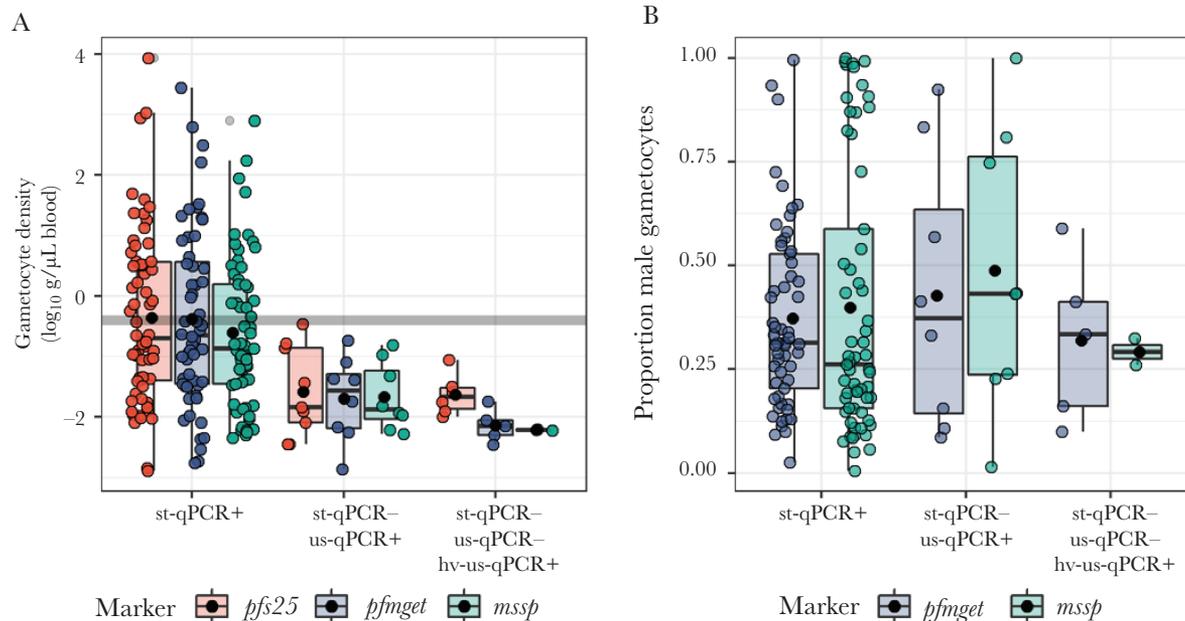


Figure 1. Gametocyte densities and proportion of males in *Plasmodium falciparum*-positive individuals detected by molecular detection methods for asexual parasites. *A*, Female and male gametocyte densities by *pfs25* (red), *pfmget* (blue), or *mssp* (green) were quantified in 93 gametocyte-positive *P. falciparum*-infected individuals detected by 3 different diagnostic methods of increasing sensitivity [3]: (1) st-qPCR, fingerprick sampling combined with standard 18S qPCR; (2) us-qPCR, fingerprick sample and positive only by ultrasensitive *varATS* qPCR; and (3) hv-us-qPCR, high-volume blood sampling combined with ultrasensitive qPCR. Mean (black circles) and median (black bars) gametocyte densities are shown. The horizontal grey line represents the gametocyte density of 1 female and 1 male gametocyte per 2.5 μL blood (0.4 gametocyte/ μL), the density required to infect a mosquito. *B*, Proportion of males by *pfs25/pfmget* (blue) or *pfs25/mssp* qRT-PCR (green) with means (black circles) and medians (black bars) presented in 71 *pfs25/pfmget* and 66 *pfs25/mssp*-positive gametocyte carriers with >10 gametocytes (female + male) gametocytes/800 μL blood. Abbreviation: qRT-PCR, quantitative reverse transcription polymerase chain reaction.

lower by *pfs25/pfmget* qRT-PCR (0.38; 95% CI, 0.24–0.52; P value $< .001$) and *pfs25/mssp* qRT-PCR (0.24; 95% CI, 0.10–0.39; P value $< .001$) (Figure 2A). IFA overestimated sex ratios by an average 0.06 (95% limits of agreement [LoA], -0.13 to 0.25) or 0.20 (95% LoA, -0.03 to 0.42) compared to *pfs25/pfmget*-qRT-PCR or *pfs25/mssp*-qRT-PCR (Figure 2C).

Proportion of Males in *P. falciparum* Infections by Detection Methods for Asexual Densities

Gametocyte densities and proportion of males are important determinants for human-to-mosquito transmission [21]. The proportion of males varied substantially among gametocytemic individuals and ranged from strong female bias to strong male bias (Figure 1B). The mean proportion of males did not significantly differ between *pfs25/pfmget* (0.39; 95% CI, 0.33–0.44) and *pfs25/mssp* qRT-PCR (0.41; 95% CI, 0.34–0.49; P value = .15). Gametocyte sex ratio was male biased in 30% (21/71) of gametocyte carriers by *pfs25/pfmget* qRT-PCR and was similar for *pfs25/mssp* qRT-PCR (Figure 1B). Age was no predictor for total gametocyte density (Supplementary Table 6). We found no association between the proportion of males with total gametocyte density (P value = .45) or asexual parasite density (P value = .46) (Supplementary Table 7).

Predicting the Likelihood of Transmission Using a Poisson Model

A mosquito bloodmeal consists on average of 2–3 μL blood [28] and must contain at least 1 female and 1 male gametocyte (corresponding

to 0.4 gametocytes/ μL assuming a 2.5 μL bloodmeal) for mosquito infection. We predicted the probability of potential transmitters among 161 study participants, *P. falciparum* positive by any diagnostic, assuming a gametocyte density of at least 1 female and 1 male gametocyte/2.5 μL blood (Figure 3A). The probability of carrying a minimum of 1 gametocyte of either sex was highest for *P. falciparum* infections detected by standard 18SrRNA qPCR (0.29) (Figure 3B). In *P. falciparum* infections exclusively detected by us-qPCR, the probability was substantially lower (0.02), and close to zero in *P. falciparum* infections detected exclusively by hv-us-qPCR (0.007) (Figure 3B). When translating these probabilities into numbers of gametocyte carriers who can potentially transmit, 25 of 87 individuals *P. falciparum* positive by standard qPCR were predicted to carry at least 1 female and 1 male gametocyte in an average mosquito bloodmeal. Among the 15 and 59 *P. falciparum* infections detected exclusively by us-qPCR or hv-us qPCR, 0.2 or 0.4 infections were estimated to carry at least 1 female and 1 male gametocyte per 2.5 μL blood. In total, 16% (26/161) of all *P. falciparum*-positive individuals in our study community were predicted to carry the minimum required gametocyte density to infect a mosquito. Ultrasensitive detection methods accounted only for 0.4% (0.6/161) of these.

DISCUSSION

The recent development of highly sensitive male gametocyte-specific biomarkers permits the discrimination and relative

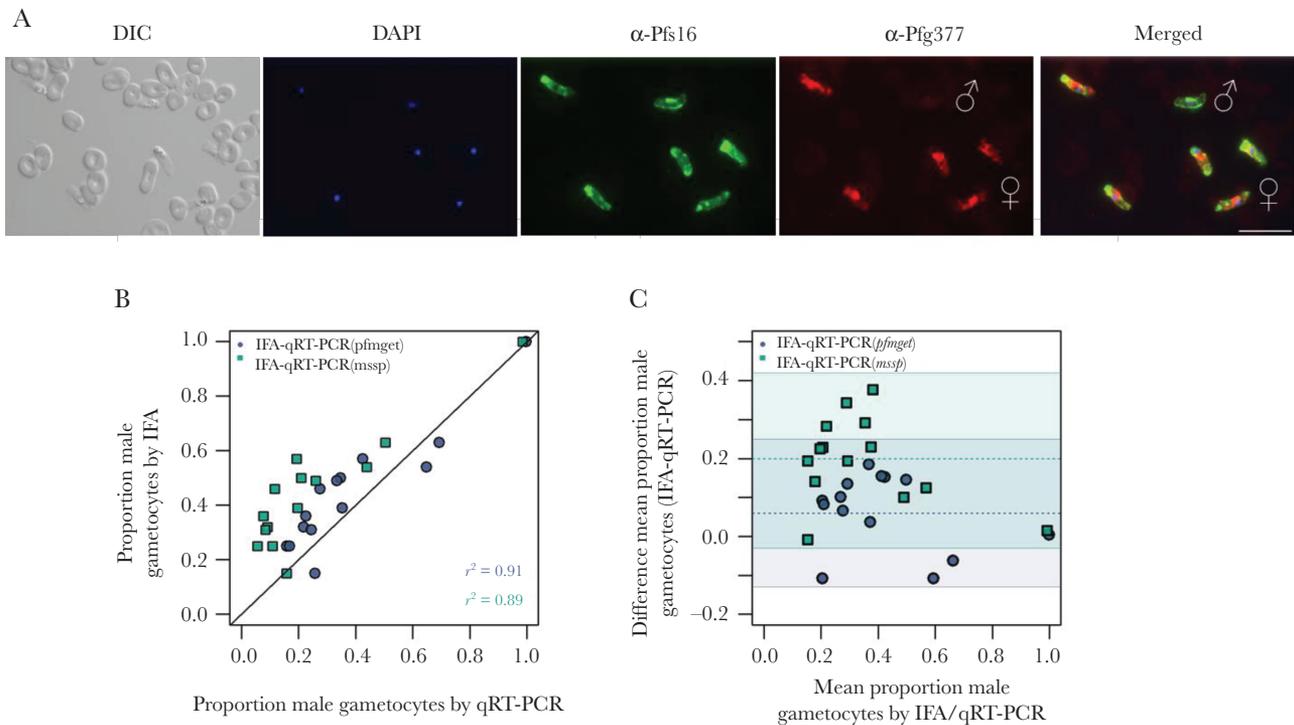


Figure 2. Proportion of males determined by immunofluorescence assay (IFA) and qRT-PCR in 14 asymptomatic study participants from Papua New Guinea. *A*, Immunofluorescent staining of MACS-enriched *Plasmodium falciparum* gametocytes from a field sample. Thick smears of enriched gametocytes were methanol-fixed and stained with α -Pfs16 and α -Pfg377 antibodies. α -Pfs16 stains female and male gametocytes and α -Pfg377 is female specific. The proportion of male gametocytes was calculated as the difference in α -Pfs16- and α -Pfg377-labelled gametocytes. Nuclei were stained with DAPI. Scale bar represents 10 μ m. *B*, Correlation of proportion of males determined by IFA and *pfs25/pfmget* qRT-PCR (blue circles) or by IFA and *pfs25/mssp* qRT-PCR (green squares) in 14 samples with >10 gametocytes/slide. Proportion of males correlated well among IFA and *pfs25/pfmget* qRT-PCR (Pearson $r^2 = 0.91$; $P < .001$) and IFA and *pfs25/mssp* qRT-PCR (Pearson $r^2 = 0.89$; $P < .001$). *C*, Agreement of proportion of males by IFA and *pfs25/pfmget* qRT-PCR (blue circles) and by IFA and *pfs25/mssp* qRT-PCR (green squares) in the same samples as in (*B*). Mean difference (dashed lines) with 95% limit of agreement (LoA) (shaded area) between both methods are indicated. Mean difference IFA/*pfs25/pfmget* qRT-PCR 0.06 (95% LoA, -0.13 to 0.25) and IFA/*pfs25/mssp* qRT-PCR 0.20 (95% LoA, -0.03 to 0.42). Abbreviations: DAPI, 4',6-diamidino-2-phenylindole; DIC, differential interference contrast; qRT-PCR, quantitative reverse transcription polymerase chain reaction.

quantification of female and male gametocytes at low parasite densities [15, 16]. We optimized the sensitivity of gametocyte detection by enriching transcripts from a large volume of blood, which allowed us to assess gametocyte prevalence, density, and sex ratio even in natural ultralow-density *P. falciparum* infections with parasite densities below 1 parasite/ μ L, which were below the detection limit of standard qPCR [3]. Moreover, we performed the first ever direct comparison of gametocyte sex ratio estimates by cytological counts on IFA slides and molecular methods, confirming the validity of molecular estimates.

Gametocyte density is the key determinant of the likelihood of onward transmission to mosquitoes. Highly sensitive molecular gametocyte diagnostics can detect gametocytes at densities well below the density plausibly allowing transmission [8, 21, 29]. Quantification of gametocytes at ultralow densities is challenging. By concentrating RNA from a large volume of blood we increased analytical sensitivity, allowing detection of gametocyte densities that were too low to be detectable in fingerprick samples. This specific detection of low-density infections

effected lower mean female gametocyte densities in our study compared to a previous study from Papua New Guinea [12].

Particularly at low gametocytemia, the gametocyte sex ratio may become a critical determinant of transmission success [4, 18, 21]. Sex ratios between carriers varied considerably in this subset of individuals, ranging from strong male bias (supportive of transmission) to near absence of males (making onward transmission highly unlikely). Highly variable sex ratios in our data may derive from stochastic effects at low abundance of gametocyte-specific transcripts. We did not observe an effect of *P. vivax* coinfection, asexual parasite density, or total gametocyte density on sex ratio, although the latter was observed previously [19, 21]. Mean sex ratios in our study community are comparable with sex ratios determined in asymptomatic gametocyte carriers from Mali and Kenya [15]. In contrast, our ratios seem high with respect to studies conducted in symptomatic malaria patients in Nigeria [30, 31].

A model applied to our data predicted that bloodmeals of an average 2.5 μ L blood from 16% of *P. falciparum*-positive individuals carry at least 1 female and 1 male gametocyte. Densities

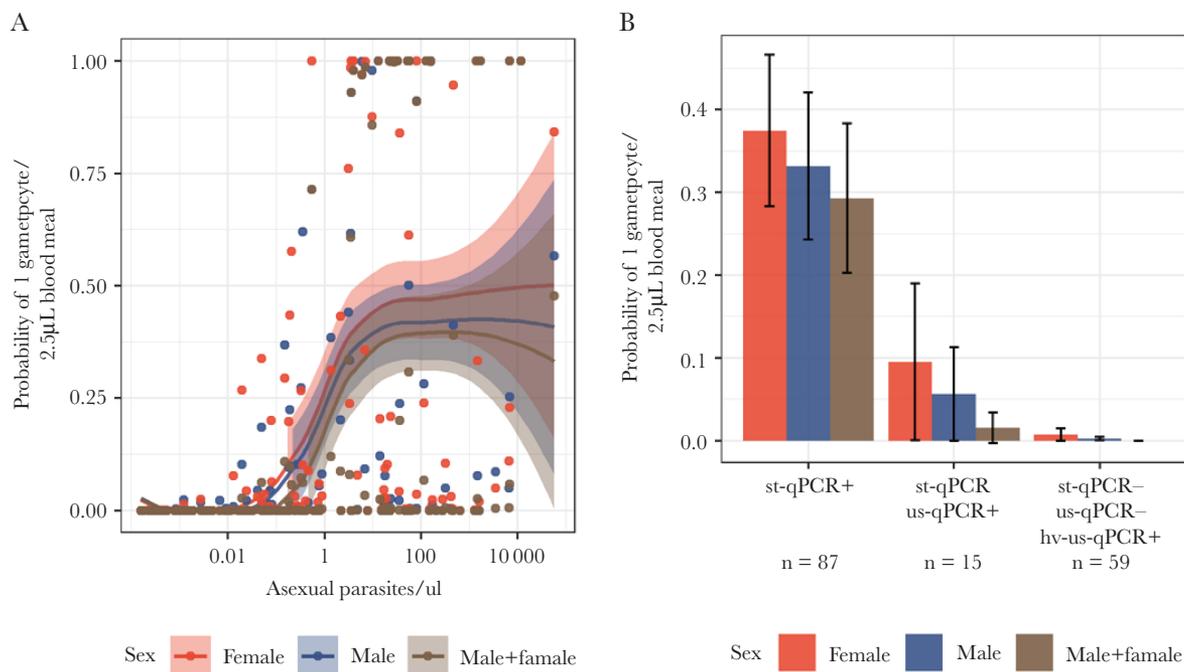


Figure 3. Transmission probability model. *A*, The mean probability of carrying at least 1 female and 1 male gametocyte/2.5 μ L blood, the minimum density required to infect a mosquito, was predicted for 161 *Plasmodium falciparum*-positive individuals using a Poisson model. Each circle represents the probability of a *P. falciparum*-positive individual with at least 1 female (red) or 1 male (blue) or 1 female and 1 male (brown) gametocyte per 2.5 μ L blood. Predicted mean probabilities (lines) and standard errors (shaded areas) are shown. *B*, Bars represent mean probabilities and 95% confidence interval (black vertical lines) of *P. falciparum*-positive individuals with at least 1 female (red) or 1 male (blue) or 1 female and 1 male gametocyte (brown) per 2.5 μ L blood. *P. falciparum*-positive individuals were detected by either standard (st) qPCR ($n = 87$), or additionally detected by ultrasensitive (us) qPCR in fingerprick ($n = 15$), or by us-qPCR in high-volume blood samples (hv-us-qPCR) ($n = 59$). Abbreviation: qPCR, quantitative polymerase chain reaction.

below this threshold are highly unlikely to be transmissible, even in individuals with very little prior malaria exposure (and thus immunity) and high numbers of examined mosquitoes [29]; thus we conclude that only a very low transmission risk would arise from 84% of all *P. falciparum*-positive individuals. This, however, applies to gametocyte counts observed at the time of sampling. Fluctuating parasite and gametocyte densities might transiently reach levels infectious to mosquitoes at a later time point [9]. If female and male gametocytes aggregate [32] or specifically cluster in the subdermal capillaries [33], average densities of gametocytes in venous blood may underestimate transmission potential and a chronic, low-density infection may still present a considerable reservoir for ongoing transmission. Following this hypothesis, even ultralow-density gametocyte carriers could contribute to transmission. However, both hypotheses for gametocyte clustering are currently speculative without any direct evidence.

Our male markers showed good correlation but imperfect agreement. One possible explanation is low-level expression of *mssp* also in schizonts, as previously demonstrated [25]. Thus, *mssp* might not exclusively target mature stage V gametocytes, whereas *pfmget* expression was 100 000-fold increased in gametocytes over asexual parasites [34]. Another explanation could be marker-specific differences in the expression-level profile at

the time of sampling. Time-course transcription profiles of *pfs25* and *pfmget* conducted on gametocytes from NF54 cultures suggested that *pfmget* transcripts declined slightly between day 7 and day 16 after commitment [15]. *mssp* transcript levels were not measured in that earlier study, therefore it cannot be ruled out that differential expression patterns contribute to imperfect agreement between these markers in our field samples. The observation of higher gametocyte prevalence by *pfs25* compared to both male markers could be explained by a female-biased sex ratio in natural infections and a higher number of *pfs25* transcripts/gametocyte [35]. Because *pfs25*-positive *pfmget*-negative samples typically had very low gametocyte densities (<0.01 gametocytes/ μ L) and were likely to be incompatible with transmission, we hypothesize that detecting and quantifying male gametocytes may allow valuable predictions of onward transmission potential. Future studies that use molecular assays in combination with MFA should test this hypothesis.

Any epidemiological interpretation of our data will be influenced by the diagnostic procedures, for example choice of molecular markers or conversion of gametocyte transcripts into gametocyte counts, which has been improved by using sex-specific gametocyte trendlines as standards for directly converting Ct values into female and male gametocyte densities. The abundance of gametocyte-specific transcripts may vary

between individual gametocytes, across gametocyte stages, and over the circulation period of stage V gametocytes. A previous study observed limited variation in *pfs25* transcript levels between parasite lines in vitro [36]. However, variability may exist and affect the accuracy of gametocyte quantification. This variation can only partially be controlled for by using synchronized stage V gametocytes trendlines for quantification. To better understand such limitations, we compared qRT-PCR results to IFA counts. This showed that sex ratio estimates overall correlated well, although differences of 10%–15% were observed. Such discrepancy can be explained by very low gametocyte counts in our community samples, never exceeding 200 gametocytes/slide. As demonstrated by repeated measurements presented in a previous publication [35], substantial fluctuations can be expected at such low densities owing to stochastic effects.

In conclusion, our results show that female- and male-specific qRT-PCR assays are highly sensitive and, in combination with large blood samples, are suitable to determine gametocyte densities and sex ratios in naturally occurring low-density *P. falciparum* infections. Estimated sex ratios correlated well between molecular and cytological measures in cross-sectional samples from Papua New Guinea. With 88% of all gametocyte carriers detectable by the female marker *pfs25*, performing this assay alone seems suitable for estimating gametocyte prevalence in field studies. In contrast, gametocyte densities will be underestimated if only *pfs25* qRT-PCR is carried out and a considerable number of gametocyte infections will be detected that are unlikely to be transmissible at the moment of sample collection. Almost all infections with at least 1 female and 1 male gametocyte per 2.5 μ L blood, and thus potentially infectious, were detected by standard qPCR. Our data suggest that this standard molecular diagnostic is sufficient to detect those asymptomatic *P. falciparum* infections with the highest likelihood of contributing to human-to-mosquito transmission in our study population. Of the *P. falciparum*-infected individuals in the study community, 16% were predicted to carry gametocytes at densities permitting onward transmission. Although their infectivity to mosquitoes was not shown here, these individuals present a probable risk for continuing transmission.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

Notes

Acknowledgments. We sincerely thank all members of the communities who participated in this study. We thank the Papua New Guinea IMR Madang laboratory and administration staff and in particular the field team for their efforts in patient

recruitment. We are grateful to R.W. Sauerwein and P. Alano for providing antibodies. We thank Amanda Ross for advice and discussions during data analysis.

Author contributions. M. G. and N. H. collected, analyzed, and interpreted data; E. N. collected data; S. K. carried out field supervision and statistical analysis; L. R. carried out field project administration and supervision, and ethical clearance; K. L. provided gametocyte trendline material; T. S. analyzed and interpreted data; T. B. interpreted data; I. F. carried out conceptualization, project administration, supervision, funding acquisition, and data interpretation; M. G. wrote the original draft; all authors reviewed and/or edited the manuscript.

Financial support. This work was supported by the Swiss National Science Foundation (grant number 310030_159580 to I. F.); the World Health Organization Special Program for Research and Training in Tropical Diseases (grant number WCCPRD4426109 2016/639607 support for filed work); the Netherlands Organization for Scientific Research (grant number 016.158.306 Vidi fellowship to K. L. and T. B.); the National Health and Medical Research Council (Career Development Fellowship grant numbers GNT1161627 to L. A. R. and GNT1141441 to S. K.).

Potential conflicts of interest. All authors: No reported conflicts of interest. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

References

1. Imwong M, Hanchana S, Malleret B, et al. High-throughput ultrasensitive molecular techniques for quantifying low-density malaria parasitemias. *J Clin Microbiol* **2014**; 52:3303–9.
2. Hofmann N, Mwingira F, Shekalaghe S, Robinson LJ, Mueller I, Felger I. Ultra-sensitive detection of *Plasmodium falciparum* by amplification of multi-copy subtelomeric targets. *PLoS Med* **2015**; 12:e1001788.
3. Hofmann NE, Gruenberg M, Nate E, et al. Assessment of ultra-sensitive malaria diagnosis versus standard molecular diagnostics for malaria elimination: an in-depth molecular community cross-sectional study. *Lancet Infect Dis* **2018**; 18:1108–16.
4. Mitri C, Thiery I, Bourgoon C, Paul RE. Density-dependent impact of the human malaria parasite *Plasmodium falciparum* gametocyte sex ratio on mosquito infection rates. *Proc Biol Sci* **2009**; 276:3721–6.
5. Kumar N. Modulation of transmission success of *Plasmodium falciparum* gametocytes (sexual stages) in various species of *Anopheles* by erythrocytic asexual stage parasites. *Acta Trop* **2017**; 176:263–9.
6. Ouédraogo AL, Gonçalves BP, Gnémé A, et al. Dynamics of the human infectious reservoir for malaria determined

- by mosquito feeding assays and ultrasensitive malaria diagnosis in Burkina Faso. *J Infect Dis* **2016**; 213:90–9.
7. Schneider P, Bousema JT, Gouagna LC, et al. Submicroscopic *Plasmodium falciparum* gametocyte densities frequently result in mosquito infection. *Am J Trop Med Hyg* **2007**; 76:470–4.
 8. Gonçalves BP, Kapulu MC, Sawa P, et al. Examining the human infectious reservoir for *Plasmodium falciparum* malaria in areas of differing transmission intensity. *Nat Commun* **2017**; 8:1133.
 9. Tadesse FG, Slater HC, Chali W, et al. The relative contribution of symptomatic and asymptomatic *Plasmodium vivax* and *Plasmodium falciparum* infections to the infectious reservoir in a low-endemic setting in Ethiopia. *Clin Infect Dis* **2018**; 66:1883–91.
 10. Bousema T, Dinglasan RR, Morlais I, et al. Mosquito feeding assays to determine the infectiousness of naturally infected *Plasmodium falciparum* gametocyte carriers. *PLoS One* **2012**; 7:e42821.
 11. Tadesse FG, van den Hoogen L, Lanke K, et al. The shape of the iceberg: quantification of submicroscopic *Plasmodium falciparum* and *Plasmodium vivax* parasitaemia and gametocytaemia in five low endemic settings in Ethiopia. *Malar J* **2017**; 16:99.
 12. Koepfli C, Robinson LJ, Rarau P, et al. Blood-stage parasitaemia and age determine *Plasmodium falciparum* and *P. vivax* gametocytaemia in Papua New Guinea. *PLoS One* **2015**; 10:e0126747.
 13. Ouédraogo AL, Bousema T, de Vlas SJ, et al. The plasticity of *Plasmodium falciparum* gametocytaemia in relation to age in Burkina Faso. *Malar J* **2010**; 9:281.
 14. Abdel-Wahab A, Abdel-Muhsin AM, Ali E, et al. Dynamics of gametocytes among *Plasmodium falciparum* clones in natural infections in an area of highly seasonal transmission. *J Infect Dis* **2002**; 185:1838–42.
 15. Stone W, Sawa P, Lanke K, et al. A molecular assay to quantify male and female *Plasmodium falciparum* gametocytes: results from 2 randomized controlled trials using primaquine for gametocyte clearance. *J Infect Dis* **2017**; 216:457–67.
 16. Lasonder E, Rijpma SR, van Schaijk BC, et al. Integrated transcriptomic and proteomic analyses of *P. falciparum* gametocytes: molecular insight into sex-specific processes and translational repression. *Nucleic Acids Res* **2016**; 44:6087–101.
 17. Reece SE, Ramiro RS, Nussey DH. Plastic parasites: sophisticated strategies for survival and reproduction? *Evol Appl* **2009**; 2:11–23.
 18. Reece SE, Drew DR, Gardner A. Sex ratio adjustment and kin discrimination in malaria parasites. *Nature* **2008**; 453:609–14.
 19. Schneider P, Babiker HA, Gadalla AAH, Reece SE. Evolutionary sex allocation theory explains sex ratios in natural *Plasmodium falciparum* infections. *Int J Parasitol* **2019**; 49:601–4.
 20. Bradley J, Soumaré HM, Mahamar A, et al. Transmission-blocking effects of primaquine and methylene blue suggest *P. falciparum* gametocyte sterilisation rather than effects on sex ratio [published online ahead of print 12 February, 2019]. *Clin Infect Dis* doi: 10.1093/cid/ciz134.
 21. Bradley J, Stone W, Da DF, et al. Predicting the likelihood and intensity of mosquito infection from sex specific *Plasmodium falciparum* gametocyte density. *eLife* **2018**; 7:e34463.
 22. Delves MJ, Miguel-Blanco C, Matthews H, et al. A high throughput screen for next-generation leads targeting malaria parasite transmission. *Nat Commun* **2018**; 9:3805.
 23. Koepfli C, Mueller I. Malaria epidemiology at the clone level. *Trends Parasitol* **2017**; 33:974–85.
 24. Wampfler R, Mwingira F, Javati S, et al. Strategies for detection of *Plasmodium* species gametocytes. *PLoS One* **2013**; 8:e76316.
 25. Santolamazza F, Avellino P, Siciliano G, et al. Detection of *Plasmodium falciparum* male and female gametocytes and determination of parasite sex ratio in human endemic populations by novel, cheap and robust RTqPCR assays. *Malar J* **2017**; 16:468.
 26. Karl S, Davis TM, St-Pierre TG. A comparison of the sensitivities of detection of *Plasmodium falciparum* gametocytes by magnetic fractionation, thick blood film microscopy, and RT-PCR. *Malar J* **2009**; 8:98.
 27. Alano P, Read D, Bruce M, et al. COS cell expression cloning of Pf377, a *Plasmodium falciparum* gametocyte antigen associated with osmiophilic bodies. *Mol Biochem Parasitol* **1995**; 74:143–56.
 28. Bousema T, Drakeley C. Epidemiology and infectivity of *Plasmodium falciparum* and *Plasmodium vivax* gametocytes in relation to malaria control and elimination. *Clin Microbiol Rev* **2011**; 24:377–410.
 29. Collins KA, Wang CY, Adams M, et al. A controlled human malaria infection model enabling evaluation of transmission-blocking interventions. *J Clin Invest* **2018**; 128:1551–62.
 30. Sowunmi A, Balogun T, Gbotosho GO, Happi CT, Adedeji AA, Fehintola FA. Activities of amodiaquine, artesunate, and artesunate-amodiaquine against asexual- and sexual-stage parasites in *falciparum* malaria in children. *Antimicrob Agents Chemother* **2007**; 51:1694–9.
 31. Sowunmi A, Gbotosho GO, Happi CT, Folarin OA, Balogun ST. Population structure of *Plasmodium falciparum* gametocyte sex ratios in malarious children in an endemic area. *Parasitol Int* **2009**; 58:438–43.

32. Gaillard FO, Boudin C, Chau NP, Robert V, Pichon G. Togetherness among *Plasmodium falciparum* gametocytes: interpretation through simulation and consequences for malaria transmission. *Parasitology* **2003**; 127:427–35.
33. Nixon CP. *Plasmodium falciparum* gametocyte transit through the cutaneous microvasculature: a new target for malaria transmission blocking vaccines? *Hum Vaccines Immunother* **2016**; 12:3189–95.
34. Meerstein-Kessel L, Lee R van der, Stone W, et al. Probabilistic data integration identifies reliable gametocyte-specific proteins and transcripts in malaria parasites. *Sci Rep* **2018**; 8:410.
35. Tadesse FG, Meerstein-Kessel L, Gonçalves BP, Drakeley C, Ranford-Cartwright L, Bousema T. Gametocyte sex ratio: the key to understanding *Plasmodium falciparum* transmission? *Trends Parasitol* **2019**; 35:226–38.
36. Pett H, Gonçalves BP, Dicko A, et al. Comparison of molecular quantification of *Plasmodium falciparum* gametocytes by Pfs25 qRT-PCR and QT-NASBA in relation to mosquito infectivity. *Malar J* **2016**; 15:539.