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

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**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 µL 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/µL 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 µL mosquito bloodmeal.

**Conclusions.** Based on model estimates of female and male gametocytes per 2.5 µL 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.
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* infections are highly variable and can adapt in response to anemia, the host’s immune response, coinfesting 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 coinfectected 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 gametocytes/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/
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 log10 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 X² 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 gametocytic 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 R² = 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
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 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 lower than

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+ | st-qPCR–us-qPCR+ | st-qPCR–hv-us-qPCR+ | Any Diagnostic Method |
|----------|----------|------------------|---------------------|----------------------|
| qPCR, % (n/N) | | | | |
| Asexual positivity | 54.0 (87/161) | 9.3 (15/161) | 36.7 (59/161) | 100.0 (161/161) |
| P. vivax 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 | | | | |
| pfs25, pfmget, mssp | 44.1 (71/161) | 7.5 (12/161) | 6.2 (10/161) | 57.8 (93/161) |
| Marker pairs | | | | |
| pfs25/ pfmget | 41.0 (66/161) | 6.8 (11/161) | 5.6 (9/161) | 53.4 (86/161) |
| pfs25/mssp | 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) | | | | |
| pfs25/ pfmget | 0.56 (0.23–1.37) | 0.04 (0.01–0.10) | 0.02 (0.01–0.04) | 0.28 (0.13–0.58) |
| pfs25/mssp | 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) | | | | |
| pfs25 | 36.0 (56/161) | 5.6 (9/161) | 5.0 (8/161) | 46.6 (75/161) |
| mssp | 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) | | | | |
| pfs25 | 0.35 (0.14–0.86) | 0.02 (0.01–0.06) | 0.01 (0–0.01) | 0.16 (0.07–0.35) |
| mssp | 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) | | | | |
| pfs25 | 40.4 (65/161) | 6.8 (11/161) | 3.7 (6/161) | 50.9 (82/161) |
| mssp | 38.5 (62/161) | 5.0 (8/161) | 1.9 (3/161) | 45.3 (73/161) |
| Female gametocyte density, g/µL, geometric mean (95% CI) | | | | |
| pfs25 | 0.35 (0.15–0.82) | 0.02 (0.01–0.06) | 0.02 (0–0.03) | 0.20 (0.1–0.40) |
| Marker pair pfs25/pfmget | 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 pfs25/mssp | 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. 

*aP. falciparum-infected individuals detected by standard qPCR (st-qPCR+). 

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

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

*Data was previously published by Hofmann et al [3]. 

*Nine 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. 

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

Proportion of Males in *P. falciparum* Infections Detected 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 2C). 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 per 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
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/µL, 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 affected 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 µL blood from 16% of *P. falciparum*-positive individuals carry at least 1 female and 1 male gametocyte. Densities...
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/μL) 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

**Figure 3.** Transmission probability model. A, The mean probability of carrying at least 1 female and 1 male gametocyte/2.5 μL 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 μL 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 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.
between individual gametocytes, across gametocyte stages, and over the circulation period of stage V gametocytes. A previous study observed limited variation in pf25 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 pf25, performing this assay alone seems suitable for estimating gametocyte prevalence in field studies. In contrast, gametocyte densities will be underestimated if only pf25 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 µL 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
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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.

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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, Nato 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, Bourguin 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 gametocytæmia 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 gametocytæmia in Papua New Guinea. PLoS One 2015; 10:e0126747.
13. Ouédraogo AL, Bousema T, de Vlas SJ, et al. The plasticity of Plasmodium falciparum gametocytæmia 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, Ripjma 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.
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.
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