Sex-based differences in clearance of chronic *Plasmodium falciparum* infection

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NOTE: This preprint reports new research that has not been certified by peer review and should not be used to guide clinical practice.
Abstract

Background: Multiple studies have reported a higher prevalence of malaria infection in males compared to females. However, it remains unknown whether this is due to differences in behavioral factors or biological sex playing a direct role in the host response to the malaria parasite.

Methods and Findings: To test the hypothesis that sex-based differences in host-parasite interactions affect the epidemiology of malaria, we intensively followed a cohort of individuals living in a malaria endemic area of eastern Uganda. By performing frequent sampling, ultrasensitive quantitative PCR (qPCR), and amplicon deep sequencing, we followed P. falciparum infections over time to estimate both force of infection (FOI) and rate of clearance by sex. Prevalence of malaria infection by qPCR was 14.4% in males versus 9.2% in females (difference 5.2%, 95% confidence interval [CI] 3.8% to 6.5%). There was no evidence of differences in behavioral risk factors, incidence of malaria, or FOI by sex. In contrast, females cleared asymptomatic infections at a faster rate than males (hazard ratio [HR] = 1.82, 95% CI 1.20 to 2.75 by clone and HR = 2.07, 95% CI 1.24 to 3.47 by infection event) in multivariate models adjusted for age, timing of infection onset, and parasite density.

Conclusions: In this study, differences in P. falciparum prevalence between males and females observed in endemic settings were driven by faster clearance rates in females and not by increased infection rates in males. These findings implicate biological sex-based differences as an important factor in the host response to this globally important pathogen.
Introduction

Chronic infection with *Plasmodium falciparum*, the most common and fatal malaria parasite, can lead to morbidity for those infected and contribute to ongoing transmission. Multiple studies performed in different contexts have demonstrated that, excepting impacts of pregnancy, a greater proportion of males than females in endemic settings carry malaria parasites in their blood. It has often been postulated that these differences in parasite prevalence stem from an increased risk of males acquiring infection due to socio-behavioral factors. However, biological sex itself has been demonstrated to affect responses to other pathogens, and thus an alternative hypothesis is that the sexes have different responses to the malaria parasite once infected.

Estimating the host response to *P. falciparum* infection requires close follow up of infected individuals, sensitive detection of parasites, and the ability to distinguish superinfection, which is common in endemic areas, from persistent infection. To test the hypothesis that sex-based differences in host-parasite interactions affect the epidemiology of malaria, we intensively followed a representative cohort of individuals living in a malaria endemic area of eastern Uganda. By performing frequent sampling, ultrasensitive qPCR, and amplicon deep sequencing, we were able to accurately detect the onset of new infections and follow them over time to estimate their duration. Using these data, we show that females cleared their infections more rapidly than males, implicating biological sex-based differences as important in the host response to this globally important pathogen.

Methods

Study setting and population-level malaria control interventions

This cohort study was carried out in Nagongera sub-county, Tororo district, eastern
Uganda. Nagongera district is historically a high malaria transmission area; however, 7 rounds of indoor residual spraying (IRS) from 2014-2019 have resulted in a significant decline in the burden of malaria as previously described\textsuperscript{15}.

**Study design, enrollment, and follow-up**

All members of 80 randomly selected households with at least two children were enrolled in October 2017 using a list generated by enumerating and mapping all households in Nagongera sub-county. The cohort was dynamic such that residents joining the household were enrolled and residents leaving the household were withdrawn. Data for this analysis was collected from October 1st, 2017 through March 31st, 2019. Participants were followed at a designated study clinic open daily from 8 AM to 5 PM. Participants were encouraged to seek all medical care at the study clinic and avoid the use of antimalarial medications outside of the study. Routine visits were conducted every 28 days and included a standardized clinical evaluation, assessment of overnight travel outside of Nagongera sub-county, and collection of blood by phlebotomy for detection of malaria parasites by microscopy and molecular studies. Participants with fever (>38.0°C tympanic) or history of fever in the previous 24 hours had a thick blood smear read urgently. If the smear was positive, the patient was diagnosed with malaria and treated with artemether-lumefantrine. Study participants were visited at home every 2 weeks to assess use of long-lasting insecticidal nets (LLINs) the previous night.

**Laboratory methods**

Blood smears were performed as previously described\textsuperscript{15}. For qPCR and genotyping, we collected 200 µL of blood at enrollment, at each routine visit, and at the time of malaria diagnosis. DNA was extracted using the PureLink Genomic DNA Mini Kit (Invitrogen) and parasitemia measured using an ultrasensitive qPCR assay\textsuperscript{16}. Samples with a parasite density >= 0.1 parasites/µL blood were genotyped via amplicon deep-sequencing. All samples positive
for asexual parasites by microscopy but negative for *P. falciparum* by qPCR were tested for the presence of non-falciparum species using nested PCR.17

Sequencing library preparation

Hemi-nested PCR was used to amplify a 236 base-pair segment of apical membrane antigen 1 (AMA-1) using a published protocol18, with modifications (Supplemental Appendix). Samples were amplified in duplicate, indexed, pooled, and purified by bead cleaning.

Sequencing was performed on an Illumina MiSeq platform (250bp paired-end).

Bioinformatics methods

Data extraction, processing, and haplotype clustering were performed using SeekDeep19, followed by additional filtering20. Supplemental Figure 1 shows the full bioinformatics workflow.

Data analysis

A clone was defined as a genetically identical group of parasites. A baseline infection was defined as an infection detected in the first 60 days of observation. New infections were defined as a new clone or group of clones detected in an individual after day 60. To account for the fact that a clone might be missed in a single sample due to fluctuations in parasite density21–24, we allowed 3 "skips" in detection and classified an infection as cleared only if it was not identified in 4 contiguous samples from routine visits. Additional details are found in Supplemental Table 1.

Because polyclonal infections can occur due to co-infection (one mosquito bite transmitting multiple clones) or superinfection (multiple bites), we analyzed the data both by clone and by infection event. For analysis by clone, the appearance of each unique clone was counted as a new infection and disappearance as a clearance event. For analysis by infection
event, any new clones seen within 3 visits of the date of the first newly detected clone(s) were
grouped together and considered one new “infection event.” Clearance of infection for these
events required that all clones in the group be absent for 4 routine visits.

Data analysis was conducted in R\textsuperscript{25} and Python\textsuperscript{26}. Comparisons of proportions were
made using Poisson regression with generalized estimating equations to adjust for repeated
measures. Comparison of parasite density by sex was made using linear regression with
generalized estimating equations to adjust for repeated measures. Force of infection (FOI) was
calculated by dividing the number of new infections, including malaria episodes, by person time.
Comparisons of incidence measures were made using negative binomial regression. Hazards
for clearance of untreated, asymptomatic infections were estimated using time-to-event models
(shared frailty models fit using R package “frailtypack,” version 2.12.2)\textsuperscript{27,28}. These models
assumed a constant hazard of clearance and included random effects to account for repeated
measures in individuals. Parasite density was included in the model as a time-varying covariate.
Duration of infection in days was calculated as 1/adjusted hazard.

Results

Cohort participants and \textit{P. falciparum} infections

This analysis involved data from 477 children and adults (233 males and 244 females)
that were followed for a total of 1339 person-years (Table 1).
Table 1. Behavioral risk factors for malaria infection and measures of malaria burden in study population, stratified by age and sex

| Metric                                      | Age and gender categories | All     | < 5 years old | 5-15 years old | 16 years and older |
|---------------------------------------------|---------------------------|---------|---------------|-----------------|--------------------|
|                                             |                           | Male    | Female        | Male            | Female            |
| Number of participants, n                   |                           | 233     | 244           | 73              | 84                |
| Slept under LLIN the previous night         |                           | 54.7%   | 56.3%         | 54.1%           | 56.2%             |
| Person-years of follow up                   |                           | 324.2   | 345.4         | 87.3            | 96.7              |
| Number of overnight trips, (incidence*)     |                           | 44 (0.14)| 107 (0.31)    | 21 (0.24)       | 19 (0.20)         |
| Episodes of malaria**, (incidence*)         |                           | 11 (0.03)| 13 (0.04)     | 5 (0.06)        | 2 (0.02)          |
| Number of routine visits, n                 |                           | 4,319   | 4,583         | 1164            | 1293              |
| Prevalence of parasitemia by microscopy***   |                           | 2.9%    | 1.4%          | 1.8%            | 1.1%              |
| Prevalence of parasitemia by qPCR           |                           | 14.4%   | 9.2%          | 5.8%            | 3.7%              |

*per person-year

**Malaria includes 1 episode (female, < 5 years old), due to non-falciparum species (P. malariae)

***Parasitemia by light microscopy includes 1 episode (female, 5-15 years old) due to non-falciparum species (P. ovale)
149 of 477 participants (31.2%) included in the analysis had at least one *P. falciparum* infection detected (Figure 1).

**Figure 1. Study design.**

- 509 participants enrolled between October 1st, 2017 and March 31st, 2019
- 32 excluded for < 6 months of follow-up
- 477 participants included in primary analyses
  - 452 enrolled during initial enrollment (October 1st, 2017 through October 31st, 2017)
  - 25 enrolled during dynamic phase
- 149 participants with at least one positive qPCR result
  - 35 with low density infections unable to be genotyped
- 114 participants with at least one genotyped infection
  - 822 genotyped samples
- Clone-level data
  - 287 infections in 114 people
  - 185 baseline infections
  - 102 new infections
- Infection event-level data
  - 184 infections in 114 people
  - 99 baseline infections
  - 85 new infections

114 participants had 822 successfully genotyped samples and had infections characterized by clone and by infection event. We achieved a read count of >10,000 for 92% of genotyped samples, identifying 45 unique AMA-1 haplotypes in our population (frequencies and sequences in Supplemental Table 2). 35 samples had very low-density infections (< 1 parasite/µL) that could not be genotyped and had infections characterized at the event level only.

**Behavioral malaria risk factors and measures of malaria burden**

There was no difference in reported rates of LLIN use the previous night by sex (Table 1). Women over the age of 16 traveled overnight outside of the study area more than men (incidence rate ratio [IRR] for females vs. males = 3.39, 95% confidence interval [CI] 1.92 to
5.99), a potential risk factor for malaria exposure. In this region receiving regular rounds of IRS,
the incidence of symptomatic malaria was low in all age categories, and there was no evidence
of a difference in incidence of symptomatic malaria by sex overall (IRR for females vs. males =
1.14, 95% CI 0.37 to 3.51) or when adjusted for age (IRR = 1.24, 95% CI 0.37 to 4.13). In
contrast, prevalence of *P. falciparum* parasitemia by microscopy was 2.9% in males compared
to 1.4% in females across all age categories (difference of proportions 1.5%, 95% CI 0.87% to
2.1%), with relative differences in prevalence most pronounced in the oldest age group. Similar
findings were seen when prevalence was assessed by ultrasensitive qPCR (14.4% in males vs.
9.2% in females, 95% CI for difference of proportions 3.8% to 6.5%), again with the largest
differences seen in the oldest age group. There was no evidence for differences in parasite
density as determined by qPCR between males and females after adjusting for age as a
continuous variable (p = 0.47).

**Force of infection by age and sex**

To determine whether higher infection prevalence in males was due to an increased rate
of infection, we used longitudinal genotyping to calculate the force of infection (FOI, number of
new blood stage infections per unit time). Overall, the FOI was low, with new infections
occurring on average less than once every 5 years (Table 2).
Table 2. Molecular force of infection (FOI) by clone and by infection event, stratified by age and sex

| Molecular force of infection (FOI) | Sex    | Age category             |
|-----------------------------------|--------|--------------------------|
|                                   |        | All                      | < 5 years | 5-15 years | 16 years or older |
| By clone, ppy* (95% CI)           | All    | 0.17 (0.13-0.23)         | 0.14 (0.07-0.23) | 0.18 (0.08-0.39) | 0.19 (0.09-0.44) |
|                                   | Male   | 0.18 (0.12-0.28)         | 0.16 (0.06-0.45) | 0.19 (0.12-0.33) | 0.21 (0.09-0.48) |
|                                   | Female | 0.16 (0.09-0.30)         | 0.11 (0.02-0.49) | 0.18 (0.08-0.42) | 0.18 (0.06-0.54) |
| By event, ppy* (95% CI)           | All    | 0.14 (0.11-0.18)         | 0.09 (0.06-0.16) | 0.16 (0.08-0.30) | 0.16 (0.08-0.32) |
|                                   | Male   | 0.16 (0.11-0.22)         | 0.13 (0.07-0.26) | 0.18 (0.12-0.27) | 0.15 (0.07-0.15) |
|                                   | Female | 0.13 (0.08-0.21)         | 0.06 (0.02-0.19) | 0.14 (0.07-0.27) | 0.18 (0.07-0.43) |

*per person-year
There was no evidence for a significant difference in FOI by sex overall (IRR for females vs. males = 0.88, 95% CI 0.48 to 1.62 by clone and IRR = 0.83, 95% CI 0.52 to 1.33 by infection event). There was also no evidence for a significant difference in FOI by sex when adjusted for age category (IRR = 0.84, 95% CI 0.45 to 1.56 by clone and IRR = 0.79, 95% CI 0.49 to 1.28 by infection event).

Rate of clearance of infection and duration of infection by sex

Since females had a lower prevalence of infection but similar rate of acquiring infections compared to males, we evaluated whether there was a difference between sexes in the rate at which infections were cleared. Unadjusted hazard ratios for clearing infecting clones showed that asymptomatic infections cleared naturally (i.e., when not treated by antimalarials) at nearly twice the rate in females vs. males (hazard ratio (HR) 1.92, 95% CI 1.19 to 3.11, Table 3). In addition, new infections cleared faster than baseline infections and monoclonal infections cleared faster than polyclonal infections. Unadjusted hazard ratios for clearance of infection events (as opposed to clones) also showed faster clearance in females vs. males (HR = 2.30, 95% CI 1.20 to 4.42).
Table 3. Hazard ratios for rates of clearance of infection, by clone and by infection event

| Predictors          | Categories                  | Hazard ratio by clone (95% CI) | Hazard ratio by infection event (95% CI) |
|---------------------|-----------------------------|-------------------------------|-------------------------------------|
|                     |                             | Unadjusted          | Adjusted       | Unadjusted          | Adjusted       |
| Sex                 | Male                        | ref                | ref             | ref                | ref             |
|                     | Female                      | 1.92 (1.19-3.11)   | 1.82 (1.20 – 2.75) | 2.30 (1.20 – 4.42) | 2.07 (1.24 – 3.47) |
| Age                 | 16 years or greater         | ref                | ref             | ref                | ref             |
|                     | 5-15 years                  | 0.66 (0.39 – 1.10) | 0.81 (0.49 – 1.36) | 0.82 (0.39 – 1.74) | 1.27 (0.72 – 2.25) |
|                     | < 5 years                   | 1.64 (0.79 – 3.41) | 1.55 (0.76 – 3.17) | 2.01 (0.80 – 5.00) | 1.75 (0.87 – 3.53) |
| Complexity of infection (COI) | Polyclonal (COI > 1) | ref | -- | ref | -- |
|                     | Monoclonal (COI = 1)        | 1.63 (1.03 – 2.57) | -- | 0.95 (0.38 – 2.34) | -- |
| Infection status    | Present at baseline         | ref                | ref             | ref                | ref             |
|                     | New infection               | 1.94 (1.22 – 3.07) | 1.75 (1.05 – 2.94) | 4.66 (2.58 – 8.42) | 4.32 (2.59 – 7.20) |
| Parasite density *  |                             | 0.85 (0.69 – 1.06) | 0.81 (0.65 – 1.00) | 0.41 (0.32 – 0.51) | 0.44 (0.35 – 0.54) |

*Increasing parasite density (log10) in parasites/microliter, as measured by qPCR
Results were similar in multivariate models including age, gender, the period during which the infection was first observed, and parasite density, demonstrating faster clearance in females vs. males (HR = 1.82, 95% CI 1.20 to 2.75 by clone and HR = 2.07, 95% CI 1.24 to 3.47 by infection event). In both adjusted models, new infections cleared faster than baseline infections. Higher parasite densities were associated with slower clearance by clone and by infection event, but the effect size was larger when data were analyzed by infection event (HR = 0.44, 95% CI 0.35 to 0.54). There was no evidence for interaction between age and sex in either adjusted model.

We next estimated durations of asymptomatic infection by age and sex using results from a model that included these covariates (Figure 2). Durations of infection ranged from 103 days to 447 days by clone, and from 87 to 536 days by infection event. Males had a longer duration of infection across all age categories. Children aged 5-15 years had the longest duration of infection, followed by adults. Therefore, overall, males aged 5-15 years had the longest estimated duration of infection by either clone (447 days) or infection event (526 days).
Figure 2. Estimates of duration of infection from sex- and age-adjusted model

Estimated duration of infection in days, calculated by adjusting the point estimate of the baseline hazard by coefficients of the sex- and age-adjusted model. Error bars represent standard errors of duration obtained from variance in the model coefficients. Point estimates of duration are labeled (*).

Discussion

Previous studies have reported a higher prevalence of malaria infection in males compared to females, with the difference often ascribed to differences in exposure. By closely following a cohort of children and adults and genotyping every detected infection with sensitive amplicon deep-sequencing, we were able to estimate both the rate of infection (FOI) and duration of infection and to compare these measures by sex. We found that lower prevalence in
females did not appear to be due to lower rates of infection but rather due to faster clearance of infections. To our knowledge, this is the first study to report a sex-based difference in the duration of malaria infection.

Many prior studies of malaria incidence and/or prevalence that evaluated associations with sex in late childhood, adolescence and adulthood have found a male bias in the observed measure of burden. Overall, these studies consistently suggest that males exhibit higher incidence and/or prevalence of malaria that begins during late childhood, persisting through puberty and the majority of adulthood (excepting the years when pregnancy puts women at higher risk). One possible explanation put forward for the sex-specific difference in burden has been that males are more frequently bitten by malaria-carrying mosquitos due to behavioral differences such as working outside, not sleeping under a net, or traveling for work. In our study, however, there were no significant differences in malaria incidence or FOI by sex. We also saw no evidence of behavioral trends that would result in more infections in males; in fact, older women in our study did most of the traveling outside the study area (an area of low transmission compared to surrounding areas). We did not assess work habits as part of our study questionnaire, but the fact that we observe similar patterns in prevalence by sex in all age categories makes this a less likely explanation.

Very few studies have been conducted to explore immunological differences between males and females in their response to the malaria parasite. The Garki project found that females had higher levels of antibodies against *P. falciparum* compared to men. Additionally, the RTS,S vaccine is associated with higher all-cause mortality in girls compared to boys, and a trend toward higher risk of fatal malaria was noted in vaccinated girls compared to boys. Hormonal differences may play a role; studies in mice show that testosterone appears to downregulate the immune response to malaria. More studies are needed to elucidate the relationship between sex-based biological differences between males and females and their effects on the development of effective antimalarial immunity.
Of the variables we evaluated in addition to sex, baseline infection status and parasite density were associated with the rate of clearance of infection. Infections that were already present at the beginning of the study may have been a non-random selection of well-established asymptomatic infections that were present at baseline at a higher frequency than average because they had a fundamentally different trajectory than newly established asymptomatic infections. Higher parasite densities were also associated with slower clearance of infection in both adjusted models, but the effect was most pronounced when the data were analyzed by infection event. This may be because low-density infections that we were unable to successfully genotype were only included in the infection event analysis, and these events tended to have short durations. The inclusion of parasite density in our multivariate models did not meaningfully alter associations between sex and duration of infection, providing evidence that the sex-based differences in duration were not mediated primarily by differences in parasite density in our cohort.
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