Understanding the Drivers of Submicroscopic Malaria Infection: Updated Insights from A Systematic Review of Population Surveys

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Background: Adoption of molecular techniques to detect *Plasmodium falciparum* infection has revealed many previously undetected (yet transmissible) low-density infections in areas of low malaria transmission. In a world where numerous countries are approaching elimination, additional data from low transmission areas are required to better understand the determinants of submicroscopic infection and their potential relevance in these settings.

Methods: We updated a systematic review of studies assessing asexual *P. falciparum* prevalence by microscopy and PCR in the same population. PubMed and Web of Science databases were searched up to July 2017. Bayesian regression modelling was used to explore determinants of the size of the submicroscopic reservoir across endemic populations and define when and where submicroscopic infections are likely to be most relevant to malaria control efforts.

Findings: A total of 105 references containing 387 microscopy/PCR prevalence pairs were included. Our results highlight marked geographical variation in the proportion of submicroscopically infected individuals across settings, with the submicroscopic reservoir largest in regions with historically low levels of transmission and smaller in areas that have only more recently reduced transmission. Age was also a significant determinant, with submicroscopic infection more likely in adults than infants (0-5 years) and older children (5-15 years), although we did not observe a statistically significant influence of seasonality. Integrating these results with estimates of infectivity in relation to parasite density suggests the contribution of this submicroscopic infections to transmission across different settings is likely to be highly variable.

Interpretation: Significant variation in the prevalence of submicroscopic infection exists even across settings characterised by similar current levels of transmission. These differences in submicroscopic epidemiology potentially warrant different approaches to targeting this infected sub-group in the approach to elimination.

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Research in Context:

Evidence before this study: Cross-sectional studies of malaria prevalence in endemic populations have revealed the widespread presence of infections with parasite densities lower than the threshold of detection by microscopy. Previous systematic reviews of these surveys have highlighted that the fraction of *P.falciparum* infections missed by microscopy can be substantial (~45% of all infections detected by PCR on average), that submicroscopic infection is more likely in adults than in children (a phenomenon attributed to increased levels of immunity), and that this missed fraction (the "submicroscopic reservoir") is typically highest in areas with low transmission intensity. Although further work is required, a number of studies have also demonstrated the capacity of these submicroscopic, low density infections to infect mosquitoes and therefore contribute to onwards transmission.

Added value of this study: Although previous studies have found that infections in older, more immune individuals are more likely to be submicroscopic, in lower transmission settings where individuals generally develop less immunity, a larger proportion of infections are also typically submicroscopic. Here we present an updated systematic review to explore this apparent paradox across a range of transmission settings and define contexts in which submicroscopic infections are likely to be most relevant to malaria control efforts. Leveraging the substantial increase in the availability of malaria cross-sectional surveys reporting prevalence by molecular methods, we explore the association of submicroscopic infection with a number of novel factors – these include age (at a finer resolution than has previously been possible), seasonality, geography and historical transmission patterns. Our findings highlight striking geographical variation in the size of the submicroscopic reservoir - this variation can be explained in-part by the historical patterns of transmission characterising each area, with the submicroscopic reservoir largest in areas with historically low levels of transmission. Integrating this information with estimates of the infectivity of submicroscopic and microscopically-detectable infections allows us to better define when and where submicroscopic malaria infections may be relevant to control efforts and highlights that the contribution of these low-density infections to transmission is likely to vary substantially across different settings.

Implications of all the available evidence: Our work highlights important and material differences in submicroscopic malaria epidemiology across settings and suggests the absence of a one-size-fits-all solution for malaria control efforts targeting this infected sub-group. Whether or not the submicroscopic reservoir requires targeting in the approach to elimination will depend on the particular setting, and likely warrant different approaches if the infection is to be controlled most effectively in the approach to elimination.

Introduction

Accurate detection of malaria infection during population surveys in endemic areas is a cornerstone of effective surveillance and control of the parasite. Routinely, this is undertaken using microscopy of blood films or rapid diagnostic tests, although recent years have seen increased usage of more sensitive molecular methods in research contexts. These techniques (typically PCR-based[1]) have revealed the widespread presence of infections with parasite densities lower than the threshold of detection by routine methods[2–8].

Such "submicroscopic" infections are present across a range of different settings and populations[5,9–11]. Although rarely causative of severe symptoms, they have been associated with a number of adverse outcomes during pregnancy[12,13], as well as mild anaemia[14] and other symptoms (vomiting, jaundice etc.) in children under 10[15]. In addition to their capacity to cause low-grade disease, these infections may hold public health relevance due to their contribution to onwards transmission: though typically characterised by lower parasite densities than microscopically detectable infections, individuals with submicroscopic infections frequently harbour gametocytes (the transmissible form of the parasite) and are capable of contributing to onwards transmission[16,17]. Submicrosopically infected individuals have been shown to contribute to transmission of the parasite across both areas of high[18] and low[19] transmission intensity, as well seasonal[16] and perennial[17] settings. Additionally, a recent meta-analysis revealed the detection limit of microscopy to be higher than is typically assumed and estimated that submicroscopic infections are on average only 3-fold less infectious to mosquitoes compared with microscopy-positive individuals[20], further underscoring the potential relevance of this infected sub-group to malaria control efforts.

Despite this, our understanding of the factors influencing the size of the submicroscopic reservoir across endemic populations remains far from complete. Previous systematic reviews have found that microscopy misses on average, half of all *P. falciparum* infections compared to PCR-based methods in endemic country cross-sectional surveys[21] and that adults are more likely to harbour submicroscopic

infections than children[22]. However, whilst this relationship with age has previously been attributed to greater immunity in adults, paradoxically, in low transmission and near-elimination settings (where the extent of immunity might be expected to be minimal) the largest proportion of infections are submcroscopic[23]. Previous reviews lacked sufficient data in low and very low transmission areas to explore this paradox sufficiently and moreover, identified extensive unexplained variation in the size of the submicroscopically infected population across settings, suggesting the existence of other important factors that determine the size of the submicroscopic reservoir. For example, although the burden of submicroscopic infection is highly heterogeneous across different locations[9,24], it remains unclear whether this represents systematic variation according to geography or is reflective of other underlying location-specific characteristics.

Resolving these gaps in our understanding of submicroscopic epidemiology has material consequences for the future of malaria control. Despite mixed reports surrounding more recent progress[25], transmission is still declining in many endemic countries and the WHO has identified 21 countries that have the potential to eliminate by 2020 (http://www.who.int/malaria/areas/elimination/e2020/en/). Low transmission settings (such as those approaching elimination) frequently possess high proportions of submicroscopically infected individuals[23,26]. It remains unclear whether there is benefit in detecting and treating such cases, or whether resources are better spent elsewhere. Improving our understanding of the drivers of submicroscopic infection in these low transmission areas is therefore crucial to enable better definition of when and where submicroscopic infection is likely to occur and how the size of the submicroscopic reservoir is likely to change as areas approach elimination.

Here we update previous reviews on submicroscopic malaria infection prevalence[21,23], leveraging the increase in the usage of molecular methods over the past 5 years to explore novel determinants of submicroscopic infection prevalence. These include geographical location and historical patterns of transmission, seasonality and the role of age at a finer resolution than previously possible. These

results are then integrated with literature-based estimates of the infectivity of submicroscopic individuals to mosquitoes in order to estimate their contribution to malaria transmission across a range of different settings.

Methods

Systematic Review Update and Data Extraction

Malaria prevalence data where both microscopy and PCR based methods had been used to determine infection were compiled, updating a previous review published in 2012[23]. Searches were carried out using PubMed and Web of Science and the search terms ("PCR" OR "Polymerase Chain Reaction") AND "falciparum". Records published January 2010 - July 2017 were searched, yielding 2136 records. A further two records, unpublished at the time of screening, were also included[19,27], as were additional data collected through contacting authors of included references. Studies reporting asexual *P.falciparum* prevalence by microscopy and PCR in the same population were included. Surveys of pregnant women, where participants have been chosen on the basis of symptoms/treatment, or did not involve a population from a defined location were excluded. After screening titles and abstracts, 231 references were retained for full text evaluation, of which 60 were included. This yielded 253 new prevalence survey pairs, and 387 total when including previous reviews (which previously identified 45 relevant references)[21,23] (**Figure 1)**.

Submicroscopic infections were defined as those where infection was detectable by PCR but not by microscopy. The specificity of microscopy compared to PCR has been shown to be high (average 98.4%[21]); we therefore assume microscopy-positive individuals are also PCR-positive. In the small number of instances where the number of microscopically detected infections was higher than those identified by PCR (n = 9), the prevalence ratio was adjusted to 1– this adjustment does not qualitatively alter the results described here.

Statistical Analysis and Bayesian Linear Regression

As in previous reviews[22], data were analysed using the regression-based methodology detailed in Sharp et al[28]:

$$\mathrm{LM_i} = \mathrm{PCR_i} + \delta_i \qquad (1)$$

$$\delta_i = \delta'_i + \beta_0(\mathrm{PCR}_i - \overline{\mathrm{PCR}}) \qquad (2)$$

where LM_i = log odds of microscopy prevalence in survey *i*, PCR_i = log odds of PCR prevalence, $\overline{\mathrm{PCR}}$ = mean survey PCR prevalence, and δ_i = log odds ratio (OR) of microscopy to PCR prevalence. This formulation allows δ_i to vary between surveys, with β_0 controlling the extent of this variation. This model was fitted within a Bayesian Markov Chain Monte Carlo based framework, implemented in the statistical software package JAGS[29].

Historical and Current Regional Transmission Intensity Stratification

Surveys conducted in African settings were geolocated and prevalence estimates (aggregated to the administrative unit 1 level) from the Malaria Atlas Project[30] used to characterise current and historical transmission intensity of the region each survey belonged to. We distinguish between local malaria transmission (defined by the prevalence recorded in each survey), and malaria transmission at the regional level (reflecting broader patterns of transmission). This regional-level transmission represents the average of a heterogeneous mixture of higher and lower transmission areas, and has relevance to local transmission because factors like human movement patterns and circulating parasite genetic diversity are often similar across nearby settings in the same region, even if transmission levels differ[31,32]. We stratified each study into one of three "transmission archetypes":

- **Historically High, Currently High:** areas that have historically (defined as 15 years previous to the date of the survey) high transmission intensity (>15% slide prevalence in 2-10 year olds (*Pf*PR_{2-10})) and remain so at the time of the survey (n = 71).
- **Historically High, Currently Low:** Areas of historically high transmission intensity that have declined in recent years to low levels (<15% *Pf*PR_{2-10}) (n = 65).
- **Historically Low, Currently Low:** Areas characterised by historical and current low transmission (<15% *Pf*PR_{2-10}) (n = 28).

Where MAP estimates were unavailable (for dates earlier than 2000), it was assumed that the year 2000 was reflective of historical transmission intensity given the only recent substantial increase in

1 international financing for malaria control (an approximately twentyfold increase between 2000 and
2 2015)[33].

3 **Calculating the Contribution of Submicroscopic Infections to Onwards Transmission**

4 Estimates of comparative infectivity of microscopically-detectable vs submicroscopic infections (the
5 "infectivity ratio") are variable, ranging from 2x[34] to a 20x difference[35]. We therefore explored three
6 scenarios where microscopically detectable infections were either 2x, 5x or 20x more infectious to
7 mosquitoes than submicroscopic infections. Proportional contribution to transmission by
8 submicroscopic infections was calculated as:

9 $$\frac{LM_{-ve}}{(LM_{+ve} * \ Infectivity\ Ratio) + LM_{-ve}}$$

10 The proportional contribution to transmission is the quantity of interest: the relative infectiousness of
11 submicroscopic infections (the prevalence of which is denoted by LM_{-ve}) is therefore set to 1, and
12 $Infectivity\ Ratio$ (2x, 5x or 20x) is a multiplicative factor reflecting the fact that microscopically
13 detectable infections (prevalence denoted by LM_{+ve}) are more infectious. The equation's
14 denominator reflects total onwards transmission occurring within the population; the numerator the
15 amount of transmission attributable to submicroscopic infections. These analyses assume that that
16 submicroscopic and microscopically infected populations do not differ in other key factors likely to
17 also influence transmission (e.g. age and mosquito exposure).
18

Results

Across the 267 surveys included in our analyses that assessed infection status across a range of ages, microscopy detected on average 46.5% of all PCR-detectable infections (43.0% - 50.0%, 95% CI), although this varied **(Fig.2A)**. There was no difference in the observed relationship between PCR and microscopy prevalence when the model was fitted separately to data collated in previous reviews and the data newly extracted here **(Supp Fig.1)**. The prevalence ratio (defined as the proportion of PCR positive infections detected by microscopy) increased as malaria transmission increased, indicating a declining proportion of submicrosopically infected individuals, from between 70-80% in the areas of lowest PCR prevalence to only 10-20% in the highest prevalence areas **(Fig.2B)**. A number of more flexible model structures were also fitted to the data, although the log-linear model provided the best overall fit (**Supp Fig.3)**. Large variation still remained in the proportion of submicroscopic infections between surveys carried out in regions with similar PCR prevalence **(Fig.2C)**, pointing to additional factors beyond current transmission intensity in shaping the size of the submicroscopic reservoir. There was no statistically significant effect of sampling season after controlling for survey PCR prevalence (ANOVA, $p = 0.181$, $df = 2$, **Fig.3)**, but a statistically significant effect of PCR methodology (ANOVA, $df = 5$, $p < 0.001$, **Supp Fig.4**). Scanning a higher number of microscopy fields to determine infection presence/absence was also significantly associated with the prevalence ratio increasing (ANOVA, $df = 1$, $p < 0.01$).

We defined 3 age-based categories: infants (0-5 years old), older children (5-15 years old) and adults (>15 years old), yielding 40, 37 and 43 prevalence survey pairs. The prevalence ratio varied significantly between age groups (ANOVA, $p < 0.001$, $df = 2$), and was significantly lower in adults (indicating a greater proportion of submicrosopic infections) compared to young children (Tukey's HSD, $p < 0.001$) and older children (Tukey's HSD, $p < 0.001$). A similar disparity was observed between older and younger children, although this difference was not significant (Tukey's HSD, $p = 0.61$). We also explored whether these differences were observed consistently across the range of transmission

settings (using survey PCR prevalence as a proxy for transmission intensity) present in the data **(Fig.4)**. Fitting the log-linear regression model separately to the data for each age group highlighted that the increased prevalence ratio observed in infants and older children compared to adults was less pronounced in higher transmission settings. For example, in high endemic areas with 70% overall PCR prevalence, the prevalence ratio for infants was predicted to be 1.42x that of adults, but 1.92x at low endemic areas with 10% overall PCR prevalence. A similar result was observed for adults and older children, suggesting genuine differences in submicroscopic epidemiology both between age groups and across transmission settings.

Grouping surveys by global region (West Africa, East Africa, South America, and Asia/Oceania) revealed significant geographical variation in the prevalence ratio (ANOVA, $p < 0.001$, $df = 3$), being lower in South American surveys compared to other regions (Tukey's HSD, $p < 0.001$ for all 3 pairwise comparisons), even after controlling for survey PCR prevalence through regression based modelling **(Fig.5).** There was no evidence that these differences were due to PCR and microscopy methodologies used in different global regions, with no substantial variation in the methodology used between regions indicating that the low prevalence ratio in South America (indicating a wider disparity in PCR and microscopy performance) was likely not occurring due to systematic differences in assay sensitivities between settings **(Supp Fig.5)**.

The majority of the South American surveys had been undertaken in areas marked by historically low transmission. We therefore investigated whether a high proportion of submicroscopic infections (low prevalence ratio) might be observed in areas around the world that had experienced similar historically low patterns of transmission. Each survey carried out in Africa (a continent for which estimates of malaria prevalence are available at a high spatio-temporal resolution) was therefore geolocated and the estimates of Historical and Current Regional Prevalence at the administrative unit 1 level collated to disentangle the effects of historical prevalence from more contemporary transmission.

Results indicated that Regional Historical Prevalence, but not Regional Current Prevalence was a significant predictor of the prevalence ratio when controlling for Survey Prevalence (ANOVA, $p < 0.001$ for both Survey Prevalence and Regional Historical Prevalence, $p = 0.73$ for Regional Current Prevalence), suggesting that historical patterns of transmission are an important determinant of the submicroscopic reservoir size. To further explore this, we classified each survey in our review from Africa into 3 transmission "archetypes" based on historical and current regional levels of transmission and fitted regression models to each. The results from these models were concordant with the results from the ANOVA, with African surveys in regions with both historically and currently low transmission (Sudan, Ethiopia and parts of Kenya and Tanzania) having on average, a lower prevalence ratio (more submicroscopic infections) compared to other low-endemic areas in Africa where historical transmission has been high **(Fig.6)**. There was no evidence of systematic differences in the PCR and microscopy methodologies in different transmission archetypes **(Supp Fig.6)** and the observed results were robust to the choice of High/Low threshold **(Supp Fig.7)**.

Integrating these results with estimates of comparative submicroscopic infectivity to mosquitoes, we estimate that in transmission settings characterised by historically low levels of transmission, submicroscopically infected individuals could account for 17.5% to 68.0% of onwards transmission in low prevalence settings (defined as <= 5% survey prevalence by PCR), depending on the assumption surrounding the comparative infectivity of microscopically detectable and submicroscopic infections **(Fig.7C)**. By contrast, our results suggest the contribution of the submicroscopic reservoir to transmission is less important in settings where transmission has only recently declined **(Fig.7B)** although their contribution is not irrelevant, ranging from 7.8% to 46.0% depending on the assumed comparative infectivity.

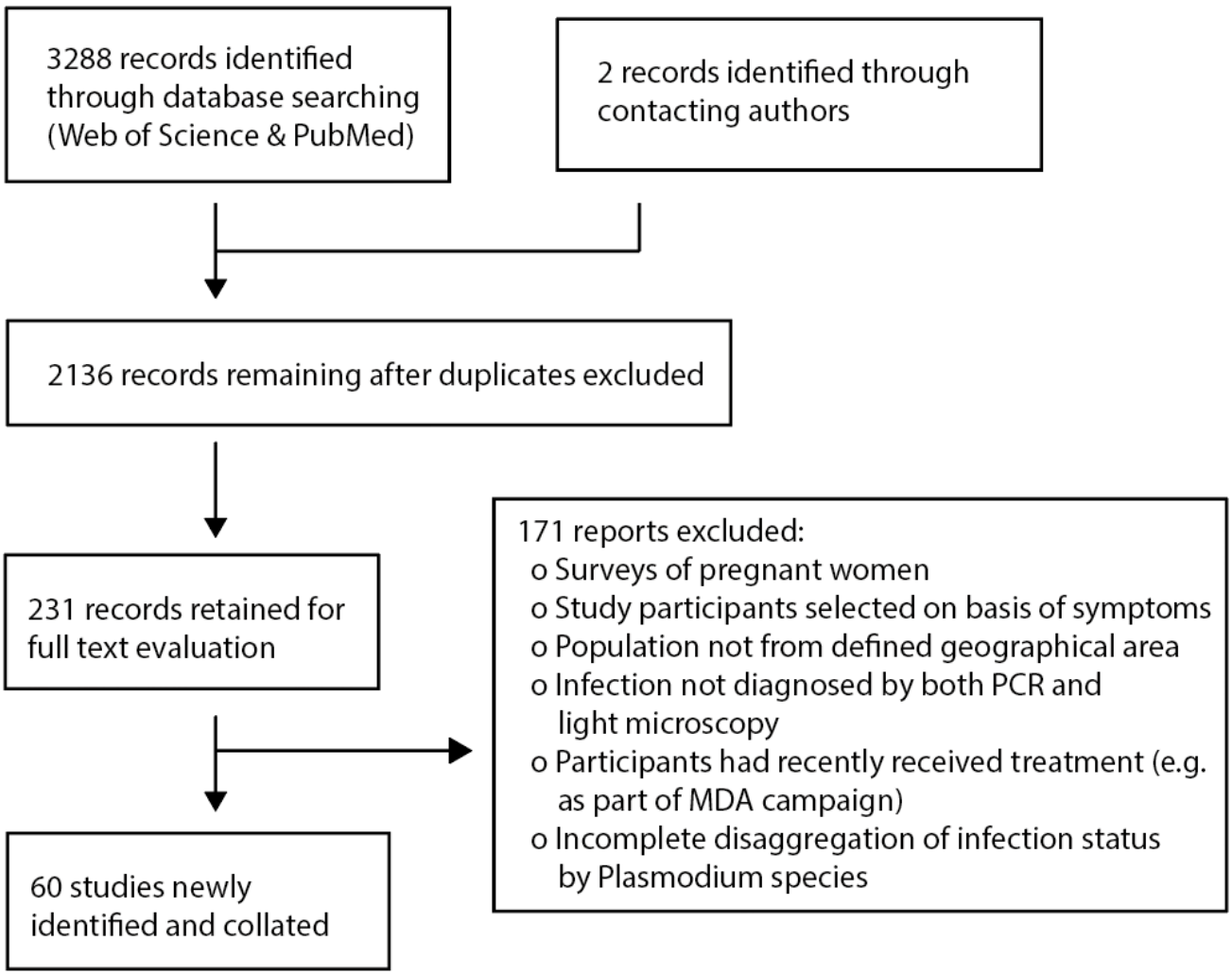

Figure 1: Systematic review overview, workflow and selection of eligible studies. Searches for malaria prevalence data where infection status had been determined using both microscopy and PCR based methods. 51 studies were included in the formal analyses, including two references unpublished at the time of screening and a limited amount of additional data collected through contacting study authors.

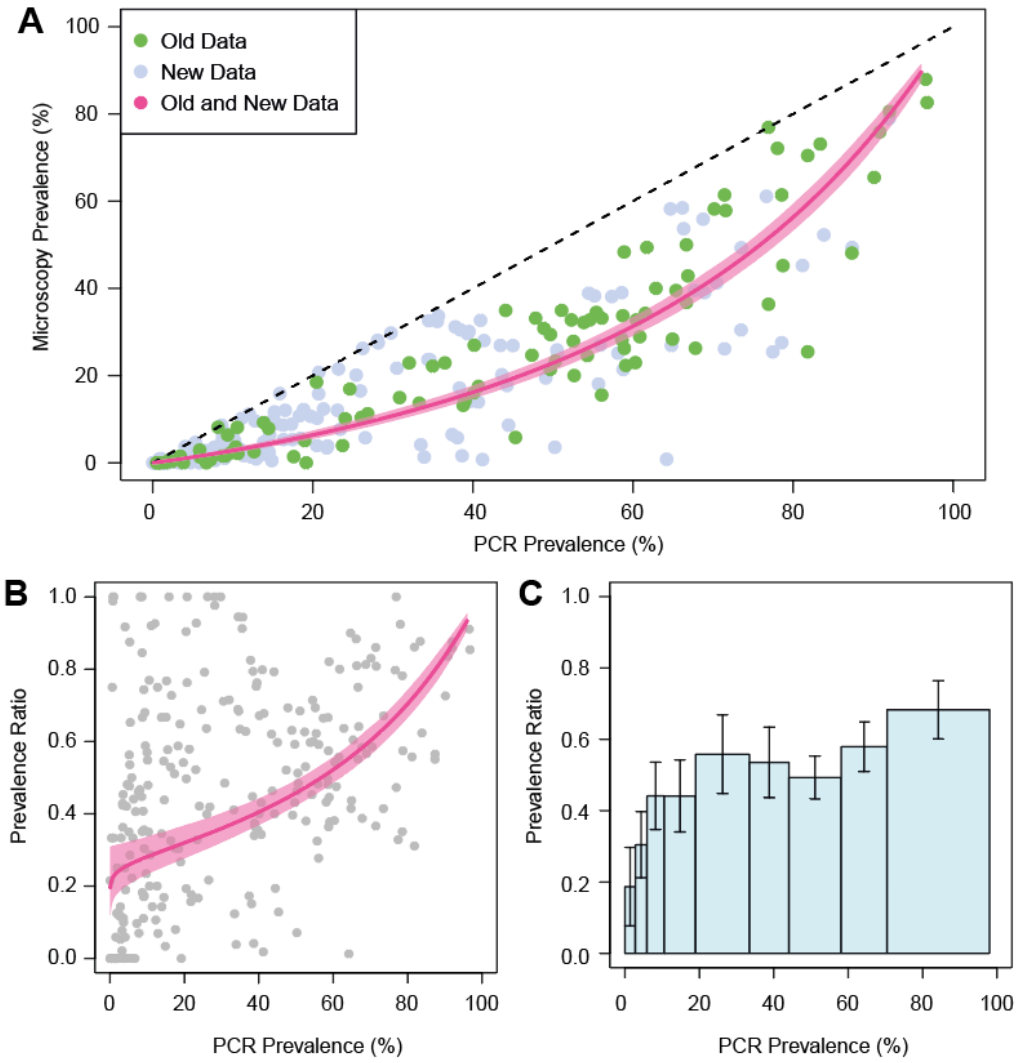

Figure 2: Prevalence of infection by PCR versus microscopy in 282 prevalence survey pairs and model fits. Bayesian Markov chain Monte Carlo methods were used to fit a linear relationship between PCR prevalence and microscopy prevalence on the log odds scale. **(A)** Microscopy and PCR prevalence (n = 267) in surveys identified in this updated systematic review (light purple points, n = 170) and in previous reviews (green points, n = 97), with the fitted model relationship (pink line) and the 95% credible interval of the mean (pale pink shaded area). **(B)** The prevalence ratio (the proportion of PCR positive individuals also detectable by microscopy) according to underlying PCR prevalence for each of the 282 survey microscopy-PCR pairs (grey points) used to fit the full model. The estimated average prevalence ratio (pink line) and 95% credible interval of the mean (pale pink shaded area) also shown**(C)** Empirically estimated microscopy sensitivity. Survey pairs were categorised into 9 categories of equal size and mean microscopy sensitivity empirically estimated for each category. Error bars represent the binomial 95% confidence interval.

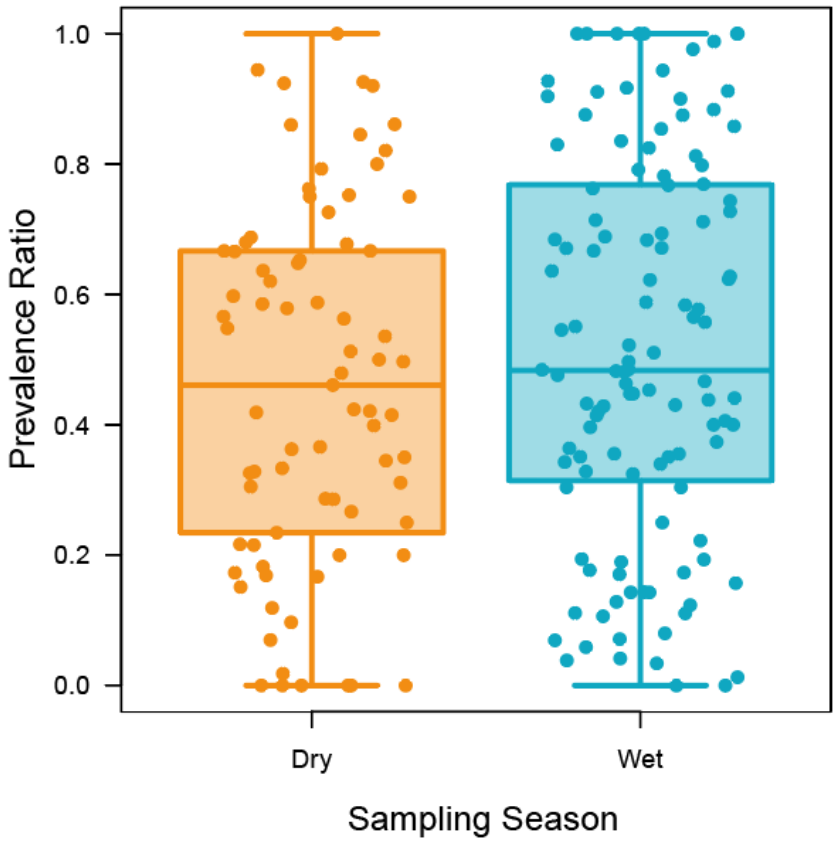

Figure 3: Comparing the prevalence ratio across different sampling seasons. Where available, information from references on the seasonal timing of the sampling was extracted and collated. Presented are boxplots of the prevalence ratio (defined as the ratio of microscopically detectable infections and PCR detectable infections, with a lower prevalence ratio indicating a higher proportion of individuals with submicroscopic infections) stratified by sampling season (n = 77 for dry season sampling, and n = 116 for wet season sampling), including also the raw datapoints (coloured circles).

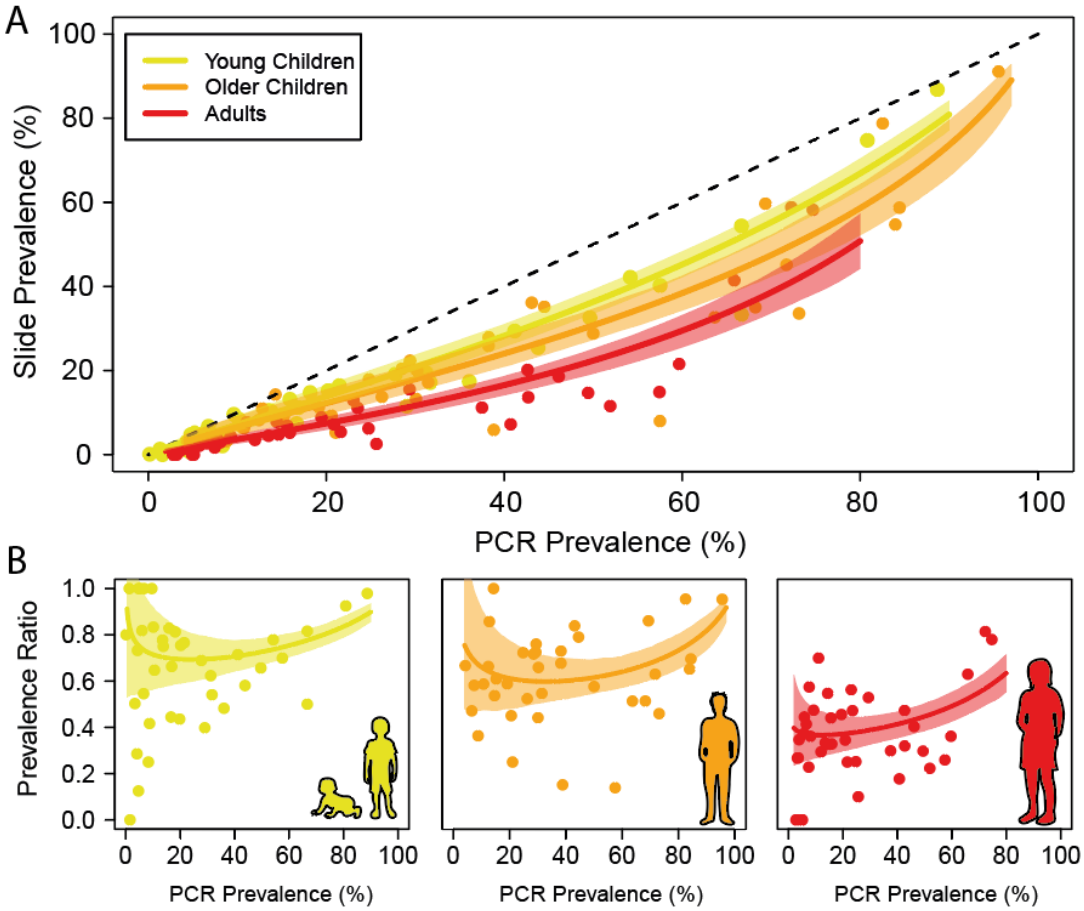

Figure 4: The influence of age on submicroscopic parasite carriage. Separate models were fitted where age disaggregated data were available to assess whether the prevalence of infection by microscopy compared to PCR varied with age. **(A)** Age disaggregated prevalence survey data for young children (0-5 years old, yellow points, **n =** 40**)** older children (5-15 years old, orange points, **n** = 37**)** and adults (red points, **n** = 43**)** with the fitted model relationship (coloured line) and 95% credible interval for each (shaded area). **(B)** The prevalence of microscopy compared to PCR (the prevalence ratio) in surveys where age-disaggregated data (dots) were available by age group, showing the fitted model relationship (coloured lines) and the 95% credible interval (shaded areas).

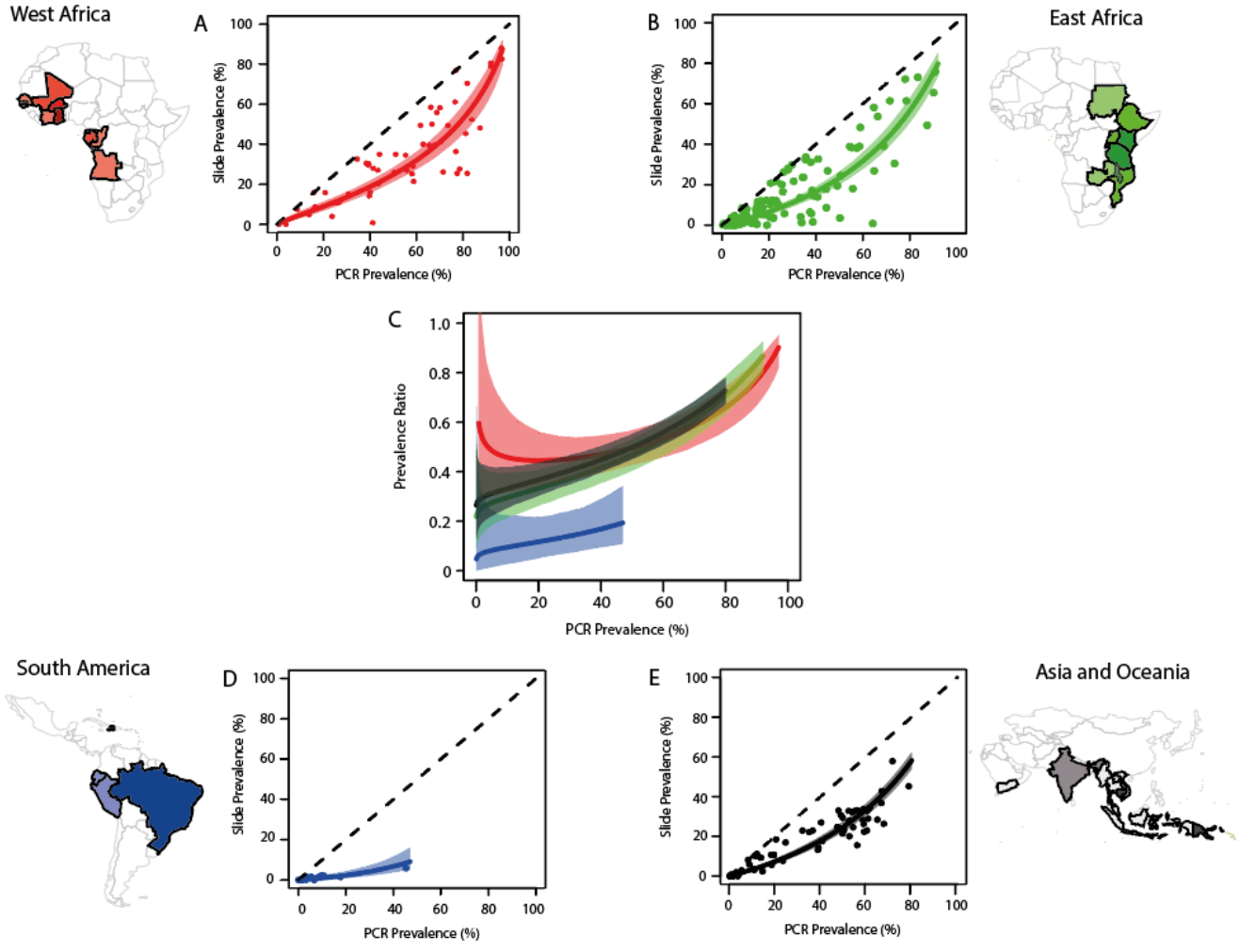

Figure 5: Global variation in the prevalence ratio and the relative size of the submicroscopic reservoir. Microscopy and PCR prevalence in included surveys (points), the model-fitted relationship (coloured line) and 95% credible interval (shaded area) for **(A)** West Africa **(n = 56)**, **(B)** East Africa **(n = 120)**, **(D)** South America **(n = 23)** and **(E)** Asia and Oceania **(n = 66)**. **(C)** The model-fitted average microscopy: PCR prevalence ratio by PCR prevalence for each of the 4 regions (coloured) line and 95% credible interval (shaded area). Intensity of colour for each country on each regional map indicates the comparative number of studies for each country, compared to other countries in that same region.

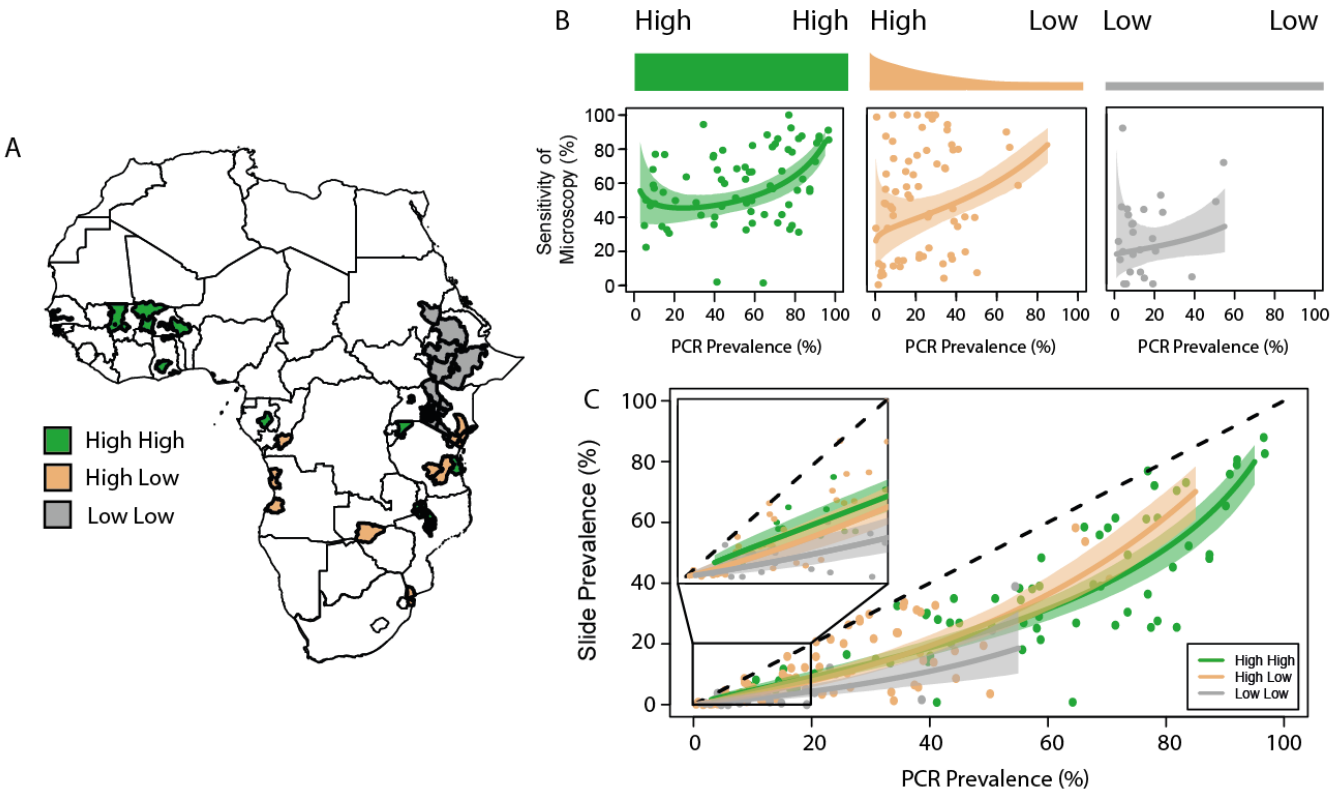

Figure 6: The effect of historical and current transmission intensity on the prevalence of submicroscopic infection in Africa. Historical estimates of malaria prevalence[36] were used to assign surveys in Africa to one of three transmission intensity archetypes, and then separate models fitted to each to explore whether variation in the present and historical levels of transmission in a region might influence the frequency of submicroscopic infection in endemic populations. **(A)** Map detailing the African countries for which prevalence surveys were identified, as well as their assigned transmission archetypes based on historical and current transmission intensity (high high= historically high and currently high; high low = historically high, currently low; low low = historically low and currently low) . **(B)** The microscopy: PCR prevalence ratio of surveys in each transmission archetype (dots, n = 72, 71 and 26 for high high, high low and low low, respectively), and the modelled average prevalence ratio (coloured line) with 95% credible interval (shaded area). **(C)** The model fitted relationship (coloured lines) and 95% credible interval (shaded areas) for each of the transmission archetypes, with a particular focus on low prevalence (<20%) settings (inset box).

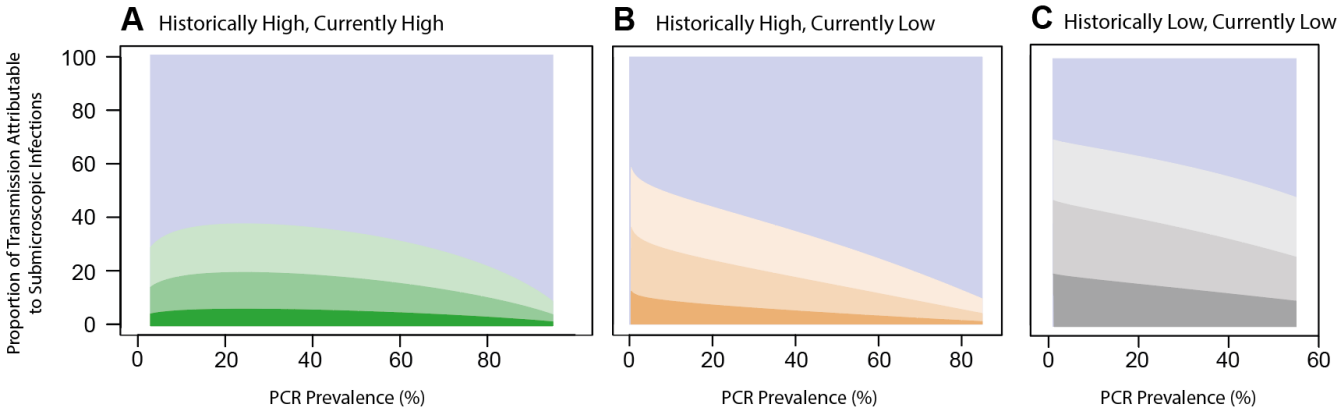

Figure 7: The potential contribution of submicroscopic infections to onwards transmission according to current and historical transmission intensity. Using our modelled relationship between PCR and microscopy prevalence (Figure 5), we estimated the potential contribution of the submicroscopic reservoir to onwards transmission for each of the transmission archetypes (A high high; B high low; C low low) if microscopic infections are either 2x, 5x or 20x more infectious than submicroscopic infections. In each panel, the purple shaded area represents the proportion of transmission attributable to microscopically detectable infections, and the lower shaded areas the contribution of submicroscopic infections for each of the 3 assumed comparative infectivities- darkest shade is 20x (i.e. that microscopically detectable infections are 20x more infectious than submicroscopic infections), intermediate shade is 5x, and lightest shade is 2x.

Discussion

Significant debate surrounds the importance of the submicroscopic reservoir to malaria control efforts, particularly whether such infections need to be targeted by interventions[37] and particularly in areas of low transmission. Disaggregating the now larger quantity of available data (282 prevalence survey pairs from 38 countries) has yielded insight into the complex relationships underlying the global pattern of submicroscopic occurrence, facilitating a more refined evaluation of when and where submicroscopic infections are likely to be most prevalent and who is most likely to harbour them. In particular, we show that the transmission gradient in the proportion of submicroscopic infections observed across all settings can be explained by differences in the historical patterns of transmission and the age structure of the infected population. Moreover, whilst previous work has generally noted the potential relevance of submicroscopic infections in low transmission settings[38], our results suggest that this relevance is likely to be highly context dependent, potentially warranting different approaches to their control depending on the setting.

Malaria prevalence across different settings varies with age[39], a feature attributed to the development of immunity after exposure, as well as age-related effects apparently independent of exposure[40,41]. As a result, increasing age and increased immunity have been associated with lower parasite densities[40,42] and our analyses confirmed a higher proportion of submicroscopically infected individuals in surveys of adults compared with children. We also found systematically larger proportions of submicroscopic infections in regions with a history of low overall malaria prevalence, a result that was observed across both South America and in regions of Africa such as Ethiopia, Sudan and parts of Kenya and Tanzania.

An important caveat to these findings is that there were insufficient data to examine the role of age and transmission history simultaneously. The average age of infection is typically higher in low transmission settings[39,43]; therefore, a greater proportion of infected individuals in all-age surveys will be adults. Our results surrounding past transmission history might then be confounded by differences in the average age of infection across these different settings. However, the age distribution of malaria

infection appears to adapt fairly rapidly to reflect changes in transmission: in an area of south-western Senegal experiencing a 30-fold reduction in malaria incidence between 1996 and 2010, there was a shift in the age distribution of cases from predominantly under-5s to a distribution that was near uniform across the population[44]. Similarly, rapid declines in malaria between 2003 and 2007 in The Gambia were shown to be accompanied by an increase in the mean age of paediatric malaria admissions from 3.9 years to 5.6 years[45]. These clinical results also extend to the prevalence of malaria infection (as assessed by microscopy) in wider populations and communities[43]. These observations suggest that the difference in infected population age structure between surveys from historically and currently low settings and those from settings where transmission has only recently declined may not be large. Therefore this confounding is unlikely to explain our finding of a lower proportion of submicroscopic infections in areas of current low transmission but where transmission has historically been high (compared to those where transmission with historically and currently low transmission).

Another potential limitation is the strong geographical bias in our transmission archetype stratification. The majority of surveys assigned to the “Low Low” archetype are from East Africa, whilst the majority of surveys in the “High High” category are from West Africa. The observed results across transmission archetypes could therefore in theory be reflecting geographical variation, rather than variation driven by past transmission history. Two strands of analysis refute this: firstly, that analysis of the submicroscopic reservoir across global regions revealed minimal difference in the prevalence ratio across West and East Africa. And secondly, that the proportion of infections which are submicroscopic in “High Low” settings (a strata also predominantly composed of studies from East Africa) was consistently lower than that observed for “Low Low” settings. These observed differences, together with the results observed for South America therefore support an effect of transmission history on the submicroscopic reservoir which is distinct from the age structure of the infected population and unlikely to be driven by geographical bias.

A number of hypotheses could explain these results, including systematic variation in asexual blood stage multiplication rate of *Plasmodium falciparum*[46] or various haemoglobinopathies and human genetic traits that have been linked to lower average parasite densities[41,47]. It is also not possible to definitively preclude systematic variation in diagnostic quality across settings, although this is perhaps unlikely: for example, a recent analysis found that whilst microscopy quality varies across settings, it does not do so systematically with transmission intensity[48]. Whilst our results highlight that PCR methodology does significantly influence the prevalence ratio (**Supp Fig.7)**, systematic variation in methodological quality across geographical (**Supp Fig.5)** and transmission archetypes **(Supp Fig.6)** settings was not observed. Interestingly, our analyses revealed no influence of seasonal effects on submicroscopic carriage, despite parasite densities having been shown to slightly rise during the rainy season even when prevalence does not change significantly (possibly due to increased occurrence of superinfection during these periods)[48,49]. This is consistent with other recent results which similarly found no difference in the submicroscopic reservoir between seasons, when directly comparing the wet and dry season submicroscopic prevalence across the same locations[20].

Variation in the extent of parasite genetic diversity between settings could also explain the observed results. Differences in the breadth and rate of immunity acquisition in settings where transmission remains high (where standing genetic diversity is typically high and individuals are exposed to a diversity of antigenically distinct parasites not previously encountered) and where transmission has historically been low (where the limited diversity of circulating strains might enable more rapid and effective development of protective immunity) could generate the patterns of submicroscopic carriage observed across different transmission archetypes. This hypothesis might go some way to explain recent results within countries that are discordant with the global pattern: recent characterisation of submicroscopic malaria carriage at three Ugandan sites with varied transmission intensity revealed little change in the extent and size of the submicroscopic reservoir across the transmission gradient at this sub-national level[10]. Furthermore, a micro-epidemiological survey in north-west Tanzania found the odds of an infection being submicroscopic actually increased with

household exposure[50]. Under an assumption of spatial circulation of strains between subnational localities (which can result in high parasite diversities even in areas of low transmission[51]), mixing of parasites between sites of varying transmission intensity but spatial proximity would result in similar extents of parasite genetic diversity[52], and by extension, submicroscopic burdens. This would be despite marked differences in levels of local transmission.

Our work suggests that the contribution of submicroscopic infections to onwards transmission is likely highly variable across settings; however, this analysis is based on the detection of asexual parasites and does not provide direct insight into gametocyte densities. The relationship between asexual parasite and gametocyte density is highly non-linear[42] and the distributions of parasite densities in the submicroscopic range can differ substantially between settings[48]. Therefore the proportion of submicroscopic infections may not relate linearly to their contribution to onwards transmission. For example, whilst a recent membrane feeding study conducted in Burkina Faso and Kenya (high transmission settings) found that 45-75% of all mosquito infections were derived from submicroscopic infections[27], only 4% of infections arose from submicroscopic individuals in a similar study carried out in Cambodia (a low transmission setting)[35]. These findings contrast with the predictions presented here and underscore the need to better resolve the relationship between submicroscopic parasite carriage, gametocyte densities, and mosquito infecitivity.

Although progress has stalled recently, substantial enthusiasm still surrounds attempts to eliminate malaria[53]. Despite this however, and despite their potential relevance, our understanding of submicroscopic infections remains far from complete. Do they represent a substantial source of transmission and threat to future progress? Or do they simply reflect the remnants of infections, capable of minimal transmission and thus less of a concern? Our work highlights important differences in submicroscopic epidemiology between settings and suggests the absence of a one-size-fits-all solution for malaria control targeting this infected subgroup. Such variation will likely warrant

different approaches to malaria control if the infection is to be controlled most effectively in the approach to elimination.

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References

1. Snounou, G. *et al.* The importance of sensitive detection of malaria parasites in the human and insect hosts in epidemiological studies, as shown by the analysis of field samples from Guinea Bissau. *Trans. R. Soc. Trop. Med. Hyg.* **87**, 649–53
2. Lamptey, H. *et al.* The prevalence of submicroscopic Plasmodium falciparum gametocyte carriage and multiplicity of infection in children, pregnant women and adults in a low malaria transmission area in Southern Ghana. *Malar. J.* **17**, 331 (2018).
3. Zhou, Z. *et al.* Assessment of submicroscopic infections and gametocyte carriage of Plasmodium falciparum during peak malaria transmission season in a community-based cross-sectional survey in western Kenya, 2012. *Malar. J.* **15**, (2016).
4. Shekalaghe, S. A. *et al.* Submicroscopic Plasmodium falciparum gametocyte carriage is common in an area of low and seasonal transmission in Tanzania. *Trop. Med. Int. Heal.* **12**, 547–553 (2007).
5. Steenkeste, N. *et al.* Sub-microscopic malaria cases and mixed malaria infection in a remote area of high malaria endemicity in Rattanakiri province, Cambodia: implication for malaria elimination. *Malar. J.* **9**, 108 (2010).
6. Baum, E. *et al.* Common asymptomatic and submicroscopic malaria infections in Western Thailand revealed in longitudinal molecular and serological studies: a challenge to malaria elimination. *Malar. J.* **15**, 333 (2016).
7. Arango, E. M. *et al.* Molecular Detection of Malaria at Delivery Reveals a High Frequency of Submicroscopic Infections and Associated Placental Damage in Pregnant Women from Northwest Colombia. *Am. J. Trop. Med. Hyg.* **89**, 178–183 (2013).
8. Mueller, I. *et al.* High sensitivity detection of Plasmodium species reveals positive correlations between infections of different species, shifts in age distribution and reduced local variation in Papua New Guinea. *Malar. J.* **8**, 41 (2009).
9. Tadesse, F. G. *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.* **16**, 99 (2017).
10. Rek, J. *et al.* Characterizing microscopic and submicroscopic malaria parasitaemia at three sites with varied transmission intensity in Uganda. *Malar. J.* **15**, 470 (2016).
11. Carmona-Fonseca, J. *et al.* Molecular Detection of Malaria at Delivery Reveals a High Frequency of Submicroscopic Infections and Associated Placental Damage in Pregnant Women from Northwest Colombia. *Am. J. Trop. Med. Hyg.* **89**, 178–183 (2013).
12. Cottrell, G. *et al.* Submicroscopic Plasmodium falciparum Infections Are Associated With Maternal Anemia, Premature Births, and Low Birth Weight. *Clin. Infect. Dis.* **60**, 1481–1488 (2015).
13. Omer, S. A. *et al.* Impact of Submicroscopic Plasmodium falciparum Parasitaemia on Maternal Anaemia and Low Birth Weight in Blue Nile State, Sudan . *J. Trop. Med.* **2019**, 1–7 (2019).
14. Ladeia-Andrade, S., Ferreira, M. U., de Carvalho, M. E., Curado, I. & Coura, J. R. Age-dependent acquisition of protective immunity to malaria in riverine populations of the Amazon Basin of Brazil. *Am. J. Trop. Med. Hyg.* **80**, 452–9 (2009).
15. Katrak, S. *et al.* Clinical consequences of submicroscopic malaria parasitaemia in Uganda. *Malar. J.* **17**, 67 (2018).
16. Ouédraogo, A. L. *et al.* Substantial Contribution of Submicroscopical Plasmodium falciparum Gametocyte Carriage to the Infectious Reservoir in an Area of Seasonal Transmission. *PLoS One* **4**, e8410 (2009).
17. Schneider, P. *et al.* Submicroscopic Plasmodium falciparum gametocyte densities frequently result in mosquito infection. *Am. J. Trop. Med. Hyg.* **76**, 470–4 (2007).
18. Ouédraogo, A. L. *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.* **213**, 90–99 (2016).
19. Tadesse, F. G. *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). doi:10.1093/cid/cix1123
20. Slater, H. C. *et al.* The temporal dynamics and infectiousness of subpatent Plasmodium falciparum infections in relation to parasite density. *Nat. Commun.* **10**, 1433 (2019).
21. Okell, L. C., Ghani, A. C., Lyons, E. & Drakeley, C. J. Submicroscopic Infection in Plasmodium falciparum –Endemic Populations: A Systematic Review and Meta-Analysis. *J. Infect. Dis.* **200**, 1509–1517 (2009).
22. Okell, L. C. *et al.* Factors determining the occurrence of submicroscopic malaria infections and their relevance for control. *Nat. Commun.* **3**, (2012).
23. Okell, L. C. *et al.* Factors determining the occurrence of submicroscopic malaria infections and their relevance for control. *Nat. Commun.* **3**, 1237 (2012).
24. Idris, Z. M. *et al.* High and Heterogeneous Prevalence of Asymptomatic and Sub-microscopic Malaria Infections on Islands in Lake Victoria, Kenya. *Sci. Rep.* **6**, (2016).
25. WHO. WHO | World malaria report 2017. *WHO* (2018).
26. Lin, J. T., Saunders, D. L. & Meshnick, S. R. The role of submicroscopic parasitemia in malaria transmission: what is the evidence? *TRENDS Parasitol.* **30**, 183–190 (2014).
27. Gonçalves, B. P. *et al.* Examining the human infectious reservoir for Plasmodium falciparum malaria in areas of differing transmission intensity. *Nat. Commun.* **8**, 1133 (2017).
28. Sharp, S. J. & Thompson, S. G. Analysing the relationship between treatment effect and underlying risk in meta-analysis: comparison and development of approaches. *Stat. Med.* **19**, 3251–3274 (2000).
29. Hornik, K., Leisch, F., Zeileis, A. & Plummer, M. *JAGS: A Program for Analysis of Bayesian Graphical Models Using Gibbs Sampling*.
30. Bhatt, S. *et al.* The effect of malaria control on Plasmodium falciparum in Africa between 2000 and 2015. *Nature* **526**, 207–211 (2015).
31. Tatem, A. J. & Smith, D. L. International population movements and regional Plasmodium falciparum malaria elimination strategies. *Proc. Natl. Acad. Sci. U. S. A.* **107**, 12222–7 (2010).
32. Tessema, S. *et al.* Using parasite genetic and human mobility data to infer local and cross-border malaria connectivity in Southern Africa. *Elife* **8**, (2019).
33. World Health Organisation. WHO | World Malaria Report 2014. (2015).
34. Coleman, R. E. *et al.* Infectivity of asymptomatic Plasmodium-infected human populations to Anopheles dirus mosquitoes in western Thailand. *J. Med. Entomol.* **41**, 201–8 (2004).
35. Lin, J. T. *et al.* Microscopic *Plasmodium falciparum* Gametocytemia and Infectivity to Mosquitoes in Cambodia. *J. Infect. Dis.* **213**, 1491–1494 (2016).
36. Bhatt, S. *et al.* The effect of malaria control on Plasmodium falciparum in Africa between 2000 and 2015. *Nature* **526**, 207–211 (2015).

37. Lin, J. T., Saunders, D. L. & Meshnick, S. R. The role of submicroscopic parasitemia in malaria transmission: what is the evidence? *Trends Parasitol.* **30**, 183–190 (2014).
38. Cotter, C. *et al.* The changing epidemiology of malaria elimination: new strategies for new challenges. *Lancet* **382**, 900–911 (2013).
39. Carneiro, I. *et al.* Age-patterns of malaria vary with severity, transmission intensity and seasonality in sub-Saharan Africa: a systematic review and pooled analysis. *PLoS One* **5**, e8988 (2010).
40. Rodriguez-Barraquer, I. *et al.* Quantification of anti-parasite and anti-disease immunity to malaria as a function of age and exposure. *Elife* **7**, (2018).
41. Drakeley, C., Gonçalves, B., Okell, L. & Slater, H. Understanding the Importance of Asymptomatic and Low-Density Infections for Malaria Elimination. in *intechopen.com* (2018).
42. Koepfli, C. *et al.* Blood-Stage Parasitaemia and Age Determine Plasmodium falciparum and P-vivax Gametocytaemia in Papua New Guinea. *PLoS One* **10**, (2015).
43. Griffin, J. T., Ferguson, N. M. & Ghani, A. C. Estimates of the changing age-burden of Plasmodium falciparum malaria disease in sub-Saharan Africa. *Nat. Commun.* **5**, 3136 (2014).
44. Brasseur, P. *et al.* Changing patterns of malaria during 1996-2010 in an area of moderate transmission in Southern Senegal. *Malar. J.* **10**, 203 (2011).
45. Ceesay, S. J. *et al.* Changes in malaria indices between 1999 and 2007 in The Gambia: a retrospective analysis. *Lancet* **372**, 1545–1554 (2008).
46. Murray, L. *et al.* Multiplication rate variation in the human malaria parasite Plasmodium falciparum. *Sci. Rep.* **7**, 6436 (2017).
47. Kiwanuka, G. N. Genetic diversity in Plasmodium falciparum merozoite surface protein 1 and 2 coding genes and its implications in malaria epidemiology: a review of published studies from 1997-2007. *J. Vector Borne Dis.* **46**, 1–12 (2009).
48. Slater, H. *et al.* The density, temporal dynamics and infectiousness of subpatent P. falciparum infections. *Nat. Commun. (In Press.* (2019).
49. Smith, T. *et al.* Absence of seasonal variation in malaria parasitaemia in an area of intense seasonal transmission. *Acta Trop.* **54**, 55–72 (1993).
50. Mosha, J. F. *et al.* Epidemiology of subpatent Plasmodium falciparum infection: implications for detection of hotspots with imperfect diagnostics. *Malar. J.* **12**, (2013).
51. Roh, M. E. *et al.* High Genetic Diversity of Plasmodium falciparum in the Low-Transmission Setting of the Kingdom of Eswatini. *J. Infect. Dis.* **220**, 1346–1354 (2019).
52. Bogreau, H. *et al.* Genetic diversity and structure of African Plasmodium falciparum populations in urban and rural areas. *Am. J. Trop. Med. Hyg.* **74**, 953–9 (2006).
53. Chen, I., Cooney, R., Feachem, R. G. A., Lal, A. & Mpanju-Shumbusho, W. The Lancet Commission on malaria eradication. *Lancet (London, England)* **391**, 1556–1558 (2018).