Malaria Policy Advisory Committee Meeting

17–19 October 2017, Geneva, Switzerland
Background document for Session 2

Meeting report of the WHO Evidence Review Group on Low-Density Malaria Infections

15–16 May 2017, Geneva, Switzerland

In March 2014, WHO published recommendations on the use of malaria diagnostics in low transmission settings. Malaria microscopy and antigen-detecting rapid diagnostic tests (RDTs) were recommended as appropriate tools for the diagnosis of clinical malaria and routine malaria surveillance. At that time, WHO recommended that the use of more sensitive nucleic acid amplification (NAA)-based methods should only be considered in epidemiological research and surveys aimed at mapping submicroscopic infections at low transmission intensity and potentially for identifying foci for special interventions in elimination settings. However, WHO also recommended that the use of NAA-based methods should not in any way divert resources away from core malaria prevention and control interventions and the strengthening of health care services, including the surveillance system.

In the years following the publication of these recommendations, the application of NAA-based diagnostic tools in epidemiological research and surveys has expanded and highly sensitive, non-NAA-based point-of-care-methods have been commercialized. Therefore, WHO convened a meeting to revise current recommendations based on a review of the natural history, prevalence, contribution to transmission, and ultimate public health importance of low-density *P. falciparum* and *P. vivax* infections. A report of the meeting proceedings, key conclusions and draft recommendations will be submitted to the WHO Malaria Policy Advisory Committee (MPAC) for consideration.

Conclusions of the ERG:

1. A high proportion of *P. falciparum* and *P. vivax* infections identified in cross-sectional surveys are characterized by low parasite densities undetectable by conventional RDT and microscopy. Although limited by small sample sizes, the relative frequency of low-density infections appears to be higher in low transmission settings than in high transmission ones. The presence of such infections is likely influenced by many factors, including the recent history of transmission, rates of superinfection, genetic diversity of parasites, treatment and immunity. More detailed analyses of existing data and larger datasets from low to very low transmission settings are required in order to improve estimates of the proportion and distribution of low-density infections. Data are limited, and there is great uncertainty regarding estimates in very low transmission settings. More studies are required that also consider the recent history of transmission and potential impact of residual immunity in the population.

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2. Evidence from several reports using mosquito-feeding experiments indicates that mosquitoes can be infected with low-density *P. falciparum* and *P. vivax* infections, although less efficiently than with high-density infections. For *P. vivax*, gametocyte densities closely follow those of asexual parasite stages. Transmission to mosquitoes becomes less efficient at *P. vivax* densities below the limit of detection (LOD) of expert microscopy (estimated at >10 parasites/µl), but can readily occur with infections below the LOD of field microscopy (estimated at >100 parasites/µl). For *P. falciparum*, the relation between gametocyte density transmissibility and the density of asexual parasitaemia is less predictable, and low-density infections below the detection level of expert microscopy can frequently result in mosquito infection. The outcome of experimental mosquito feeds is influenced by a variety of host, vector and parasite factors in addition to methodological factors, but their dynamic interactions are poorly understood.

3. Depending on the relative proportions of low- and high-density infections in a particular location, the role of each in overall transmission may vary considerably. Mosquito feeding experiments help to measure the infectiousness of low- and high-density infections for mosquitoes. However, there are limited data on the relative contributions of low- and high-density *P. falciparum* and *P. vivax* infections to the onward transmission to human populations at the community level. It is critically important to understand the contribution of low-density infections to malaria transmission in order to inform effective malaria control strategies.

4. Conclusive data on the natural history of low-density *P. falciparum* and *P. vivax* infections in different endemic settings remain elusive. Knowledge gaps exist in understanding the longitudinal dynamics of parasite density and infectivity in untreated chronic natural infections; identifying risk factors for carriage of low-density infections; and understanding the prospective clinical and pathological impacts of untreated low-density infections. Available evidence related to the different parasite biology of *P. falciparum* and *P. vivax* suggests that chronicity of infection is achieved through different mechanisms for the two species: antigenic variation and persistence in the blood stream for *P. falciparum*, and periodical relapses for *P. vivax*.

5. With the available evidence, it is difficult to accurately predict how the identification and treatment of low-density *P. falciparum* and *P. vivax* infections through active screen-and-treat based interventions in different endemic settings would impact transmission. Moreover, it is not possible to predict the proportion of the total infectious reservoir that would need to be detected and eliminated in order to accelerate the reduction of transmission. Intervention trials in different epidemiological settings using appropriate control interventions are warranted in order to evaluate the impact on transmission and cost–benefit of applying highly sensitive diagnostics for targeting low-density infections. Until the outcomes of such trials are available, highly sensitive diagnostics should not be part of any routine malaria control or elimination programme; their use should be limited to research purposes.

6. To improve comparability of results, better harmonization and standardization is required in the reporting of the molecular methods used for the detection, identification and quantification of malaria parasites in epidemiological surveys and research studies. Adherence to the Minimum Information for Publication of

Quantitative Real-time PCR Experiments (MIQE) guidelines for reporting quantitative PCR results, as well as the validation of nucleic acid-based amplification assays using standardized and quality controlled material (such as the WHO International Standard for *P. falciparum* DNA NAA Assays) is strongly encouraged. Until standardization is achieved, all reports should include a detailed description of the precise methods used to obtain the data being reported, including the analytical sensitivity and specificity of tests.

7. The terms "submicroscopic," "asymptomatic," and "low-density" infection are often used interchangeably in the literature, generating confusion. "Submicroscopic" generally implies parasitaemia that is below the LOD of microscopy or RDT, but detectable using molecular or other highly sensitive diagnostic methods. The use of the term "submicroscopic" for describing low-density malaria infections should be discouraged. The term "asymptomatic" is not based on parasite density and instead refers to the absence of signs and symptoms of malaria. Asympomatic malaria should be defined with respect to the absence of specific clinical manifestations and the time period evaluated in relation to infection detection. In light of these definitions, the term "low-density" infection is considered most appropriate. When parasitaemia is quantified, a clear definition of "low-density infection" should be reported (suggested: <100 parasites/µl), accompanied by a description of the method of quantification. In studies that do not quantify parasitaemia, low-density infections can be defined as those identified through highly sensitive methods but not detected using conventional diagnostics (microscopy or RDT).

8. Updating the WHO recommendations on the diagnosis of *P. falciparum* and *P. vivax* malaria in low transmission settings is required in order to clarify that WHO does not currently recommend highly sensitive RDTs, other highly sensitive non-NAA-based methods, or NAA-based methods for parasite detection in the routine management of clinical malaria and surveillance. Research is needed to document the public health benefits and cost–effectiveness of detecting and treating low-density infections in low transmission areas and/or specific population groups. In particular, potential research objectives for highly sensitive diagnostics could include epidemiological research to understand the contribution of low-density infections to transmission, border screening of immigrants or migrant populations, foci investigations including the mapping of low-density infections, and use in pregnant women for the detection and treatment of low or sequestered parasite biomass.

   To comply with the above conclusions, the WHO/GMP secretariat in consultation with the ERG Panel Members developed draft recommendations on the diagnosis of *P. falciparum* and *P. vivax* malaria in low transmission settings. These are listed below for consideration by the WHO MPAC.

   8.1. Quality-assured conventional RDT and microscopy are the recommended diagnostic tools for the confirmation and management of malaria cases and malaria surveillance, including routine health information systems and household surveys, in all epidemiological situations. Malaria cases should be reported by type of diagnostic test used.

8.2. A number of highly sensitive techniques are available that detect low-density infections (below 100 parasites/µl). Until there is evidence that the detection of low-density infections using these tools will accelerate malaria elimination, in elimination settings, these tools should only be used for research purposes.

8.3. The majority of infections with asexual parasites have gametocytes detectable by NAA methods, and there is no known benefit of routine detection of low-density gametocytes by molecular methods. All malaria infections (including those infections with low-density parasitaemia) should be considered as potentially infectious.

8.4. Presentation of NAA results should include details of the methods used for sample collection and extraction, and the equivalent quantity of blood added for the PCR reaction, as well as details of outputs in DNA copies or parasite density.

8.5. Before the role of serological assays in malaria elimination programmes can be determined, there is a need for standardization and validation of reagents (antigens and controls), assay methodologies and analytical approaches.

List of abbreviations

CHMI	controlled human malaria infection
FSAT	focal screening and treatment
HRP2	histidine rich protein 2
LM	light microscopy
LOD	limit of detection
MDA	mass drug administration
MIQE	Minimum Information for Publication of Quantitative Real-time PCR Experiments
MSAT	mass screening and treatment
NAA	nucleic acid amplification
PCR, qPCR	polymerase chain reaction, quantitative polymerase chain reaction
POC	point of care
PQ	primaquine
RDT	rapid diagnostic test

1. Background

Quality-assured light microscopy (LM) and rapid diagnostic tests (RDTs) that detect parasite proteins are the basic diagnostic tools currently recommended for the confirmation and management of suspected clinical malaria, as well as for routine surveillance of clinical cases in malaria-endemic settings [1]. After reviewing the evidence in 2013, WHO recommended that the use of more sensitive nucleic acid amplification (NAA) techniques for the detection of low-density malaria infections – i.e., those below the limit of detection (LOD) of LM or RDT – should only be considered for epidemiological research and surveys aimed at mapping low-density infections at low transmission intensity, or for identifying foci to guide intervention measures used specifically in elimination settings [1].

Since then, NAA-based detection of malaria infections has been increasingly applied in surveys and research studies using active or reactive surveillance of populations in endemic areas. The most commonly used method for NAA-based detection in these surveys is amplification of the 18S rRNA gene from finger-prick blood samples [2]. In recent years, quantitative NAA-based methods have often been utilized to quantify parasitaemia in low-density infections below the LOD of LM. For *P. falciparum*, and recently also for *P. vivax*, systematic reviews have concluded that LM misses approximately 50% of infections compared to polymerase chain reaction (PCR)-based detection of parasitaemia [3,4], although this proportion (and the absolute number of missed infections) varies considerably in different epidemiological settings. In cross-sectional surveys, the proportion of low-density infections among all detected infections is higher in low transmission areas than in high transmission areas for both species [4,5]. For *P. falciparum*, it is estimated that, in low to moderate transmission settings, low-density infections account for 20–50% of transmission to mosquitoes [5]; however, a comparable estimate for *P. vivax* is lacking.

Since the publication of WHO's recommendation on the use of malaria diagnostics in low transmission settings, there has been an increasing number of epidemiological surveys evaluating different diagnostic tools for reducing transmission through intervention strategies such as mass screening and treatment (MSAT) or focal screening and treatment (FSAT). In 2015, a WHO Evidence Review Group on mass drug administration (MDA), MSAT and FSAT concluded that current point-of-care (POC) diagnostic tests, MSAT and FSAT are not suitable interventions for interrupting malaria transmission. Funding agencies, manufacturers and researchers have been working towards developing highly sensitive RDTs with LODs similar to those of NAA-based methods. One highly sensitive RDT is now commercially available (Alere™ Malaria Ag P.f RDT, http://www.alere.com), and the manufacturer claims 10-fold higher sensitivity than conventional RDTs and easy deployment at the POC [6].

As more and more countries reduce the burden of malaria and move towards elimination, new evidence on the relevance of low-density *P. falciparum* and *P. vivax* infections in maintaining malaria transmission needs to be reviewed. Additionally, national malaria control programmes require clear guidance on the case management and reporting of low-density infections identified during surveys or as part of research studies. A research agenda is needed to better understand and predict the public health importance of low-density malaria infections and the potential impact of detecting them using highly sensitive RDTs.

2. Objectives

The specific objectives of the meeting were:

1. To review data on the natural history of low-density *P. falciparum* and *P. vivax* infections in different epidemiological settings; to evaluate implications for detectability, duration of infection, and infectivity; and to assess the relationship with symptoms of clinical malaria.

2. To describe the contribution of low-density *P. falciparum* and *P. vivax* infections to transmission at the population level, considering different levels of vectorial capacity and immunity in the population.

3. To define procedures for the case management and reporting of low-density *P. falciparum* and *P. vivax* infections identified through multiple means, e.g., reactive case detection, surveys, research, etc.

4. To review and update the WHO recommendations on the diagnosis of *P. falciparum* and *P. vivax* malaria in low transmission settings; these recommendations were endorsed by the Malaria Policy Advisory Committee in March 2014, based on the report of the 2013 ERG meeting.

5. To establish a set of research priorities and study design characteristics with which to address knowledge gaps on the relative importance of low-density infections and the public health impact of detecting them using highly sensitive diagnostic tests.

3. Process

The Global Malaria Programme / Prevention, Diagnosis and Treatment unit collaborated with Dr. Teun Bousema, Radboud University Medical Center of The Netherlands, and Prof. Chris Drakeley, London School of Tropical Medicine and Hygiene, in the planning of the ERG meeting and selection of studies to meet the specific objectives listed above. WHO commissioned three systematic reviews of the available evidence on the detectability and infectivity of low-density *P. falciparum* and *P. vivax* infections and on the clinical consequences of low-density infections. These pre-reads, together with relevant WHO reports, one unpublished study, and additional relevant published literature were shared with all participants as pre-reads prior to the meeting (Annex 2).

The reviews and background papers were presented and discussed in plenary at the meeting. This was followed by plenary discussions in thematic panel sessions on:

1. The natural history of low-density *P. falciparum* and *P. vivax* infections

2. Contribution to transmission of low-density *P. falciparum* and *P. vivax* infections

3. Clinical management and surveillance of low-density *P. falciparum* and *P. vivax* infections

The first part of the meeting concluded with presentations and discussions on potential programmatic applications of a highly sensitive diagnostic POC test, and on the highly sensitive Alere™ Malaria Ag P.f RDT by the test developers and their partners.

ERG participants were split into three working groups to address specific questions related to 1) natural history, 2) transmission, and 3) the clinical management and reporting of low-density malaria infections. The goals were to establish a set of research priorities, to review and update the current WHO recommendation on the diagnosis of *P. falciparum* and *P. vivax* malaria in low transmission settings, and to propose terms and definitions of low-density malaria infections. Rapporteurs of the working groups presented each group's findings to the whole group for further discussion and consensus-building.

The meeting report was compiled by Dr. Natalie Hofmann, based on the meeting pre-reads and the presentations and discussions held during the ERG meeting. All participants were invited to review the report and provide further input for consideration in finalizing the report.

In terms of objective 4 of the meeting, the outcomes of the working groups were considered separately by the WHO/GMP secretariat in consultation with the ERG Panel Members. A set of draft recommendations on the diagnosis of *P. falciparum* and *P. vivax* malaria in low transmission settings was elaborated for consideration by the WHO MPAC.

4. Evidence review

4.1. The natural history of low-density *P. falciparum* and *P. vivax* infections

4.1.1. Terminology and definitions of low-density malaria infections

The participants of the ERG noted the need for a clear distinction between the terms "submicroscopic," "low-density" and "asymptomatic" infection, as these terms are often used interchangeably both in the literature and in practice. "Submicroscopic" generally implies parasitaemia below the LOD of microscopy, which for *P. falciparum* is comparable to parasitaemia below the LOD of RDT. The term "asymptomatic" refers to the absence of signs and symptoms of malaria and is not based on parasite density. When used, the term should be defined with respect to specific clinical manifestations and the time period evaluated in relation to infection detection. The ERG agreed to promote the use of the term "low-density" to describe infections with low parasitaemia that can occur with and without signs and symptoms of malaria and that may or may not be detectable by LM.

For the purpose of this document, the committee chose to use <100 parasites/µl as a working definition of low-density infection, as this threshold focuses on parasitaemia that lies below the limit of detection of conventional microscopy. The committee, however, acknowledged that expert microscopists can detect parasitaemia below 100 parasites/µL; moreover, in many settings, "routine microscopy" does not necessarily achieve this level of sensitivity.

A clear description of the method applied to quantify parasitaemia and a definition of low-density infection (for example in relation to microscopy or a specific RDT) should be given whenever data are submitted for surveillance or for research publication. For the purpose of this report, the term "low-density" is used to discuss general concepts and future recommendations, while the term "submicroscopic" is used only when referring to specific analyses or publications in which malaria infections were stratified based on their detectability by LM.

4.1.2. *Parasite density in* P. falciparum *and* P. vivax *infections*

Many studies in recent years have applied molecular methods to quantify parasite density in malaria infections. A great deal of caution should be exercised when comparing parasite densities (and to a lesser extent parasite prevalence) across studies that differ in terms of (i) the method of sample collection and volume of blood sampled, (ii) the duration and conditions of sample storage, (iii) the method of nucleic acid extraction, (iv) the method used for molecular detection, and (v) the copy number of the target sequence. Nonlinearity in the efficiency of nucleic acid extraction and PCR amplification at different parasite concentrations adds further variability and uncertainty to pooled analyses of parasite density estimates. Standard material used to quantify parasitaemia through molecular methods differs among published studies and includes dilutions of target-specific plasmids, field sample DNA, or DNA from synchronized cultured parasites in the case of *P. falciparum*. Particularly for *P. vivax*, where late-stage parasites with multiple genomes are present in the blood stream, conversion between different measures of density is not straightforward (although data from South-East Asia suggest that the presence of mixed parasite stages in the blood does not cause major errors in *P. vivax* density estimates [7]). Although guidelines exist for the reporting of quantitative PCR (qPCR) experiments ("MIQE guidelines" [8]), the reviewed publications often provided insufficient details on the molecular method used for detection.

As more and more studies have applied quantitative NAA techniques for malaria diagnosis in epidemiological surveys, an increasing amount of quantitative parasite density data has become available. In preparation for the meeting, the committee reviewed studies from different endemic settings that used NAA-based methods to quantify parasite density in infected individuals in the community, without selection based on signs and symptoms of malaria or a positive malaria test result. Median *P. falciparum* density by quantitative NAA methods varied between 1 and 1300 parasites/µl. Geometric mean *P. vivax* density in the blood varied between 2 and 50 DNA copies/µl in moderate (Solomon Islands) to high transmission (Papua New Guinea) areas, and between 1 to 213 parasites/µl in low transmission areas of Colombia, Guatemala and Ethiopia.

The density distributions of microscopically detectable and submicroscopic *P. falciparum* infections overlapped in all studies reviewed, highlighting the role of chance and variations in methodology related to both LM and NAA-based techniques. In submicroscopic *P. falciparum* infections, median densities in the reviewed studies ranged from 0.1 parasites/µl to 330 parasites/µl – versus 2 to 9000 parasites/µl in microscopically detectable *P. falciparum* infections. Sufficient data to assess *P. vivax* parasite density in submicroscopic versus LM-detectable infections were only available from Papua New Guinea and Solomon Islands, where the geometric mean *P. vivax* densities ranged between 2 and 8 DNA copies/µl in submicroscopic infections and between 2 and 980 DNA copies/µl in LM-detectable infections.

4.1.3. *The proportion of low-density* P. falciparum *and* P. vivax *infections in cross-sectional surveys in different endemic settings*

Consistent with previously published findings [3–5,9] and after the inclusion of more recently published studies based on quantitative NAA methods, the two reviews presented at the meeting confirmed that low-density *P. falciparum* and *P. vivax* infections constitute a higher proportion of all infections in low transmission settings than in high transmission

settings. However, given that the absolute number of infections is small in low and very low transmission settings, the absolute number of low-density infections is also smaller than in high transmission settings. In cross-sectional surveys, among the infected population with presence of malaria parasites confirmed by a diagnostic test, the relative proportion of low-density infections was similar for *P. falciparum* and *P. vivax* across the endemicity spectrum (Table 1 shows the proportion of infections that were submicroscopic at different levels of endemicity).

Low-density infections represented at least half of the infections in all transmission settings (>57% of *P. vivax* and >51% of *P. falciparum* infections, Table 1). In addition, for *P. vivax,* a large number of individuals without current blood-stage parasitaemia were infected with hypnozoites that could not be detected. Estimates from Papua New Guinea suggest that approximately 80% of new *P. vivax* blood-stage infections originate from relapsing hypnozoites in tropical areas [10]. These estimates support the presence of a large hypnozoite reservoir in the population.

In the few areas that have monitored low-density infections over time during a period of reduction in transmission, the relative proportion of low-density *P. falciparum* and *P. vivax* infections has increased slightly over time (indicated by an increase in submicroscopic infections) [5–9 and Robinson, unpublished].

TABLE 1.
The proportion of *P. falciparum* and *P. vivax* infections that are submicroscopic at different levels of transmission. Transmission intensity is classified by malaria prevalence assessed using NAA-based techniques.
Data taken from published and unpublished studies assessing *P. falciparum* and *P. vivax* parasitaemia using NAA-based methods (Slater & Okell, Robinson, meeting pre-reads).

	Low transmission 0–10%	Moderate transmission 10–20%	High transmission >20%
P. falciparum			
Number of studies	n = 9	n = 1	n = 8
Unweighted Mean[1] (IQR)	75.0% (77.3–90.4)	not applicable	56.7% (51.4–63.6)
Weighted Mean[2] (CI_{95})	85.4% (81.5–88.7)	72.2 (67.4–76.6)	51.1% (48.7–53.5)
P. vivax			
Number of studies	n = 29	n = 20	n = 15
Unweighted Mean[1] (IQR)	82.5% (68.0–100)	72.6% (59.2–90.7)	57.2% (50.0–73.8)
Weighted Mean[2] (CI_{95})	70.7% (67.5–73.8)	72.0% (70.2–73.7)	58.1% (56.3–59.8)

[1] The unweighted mean is calculated by taking the raw average across all studies, by transmission level, of the proportion of submicropscopic infections observed in each study (independent of study size). The interquartile range is given as a measure of variability in the proportion of submicropscopic infections between studies. For *P. falciparum* only, one study was characterized as "moderate transmission" and the unweighted mean is thus not applicable.

[2] The weighted mean is calculated as an overall proportion of submicroscopic infections from accumulated data by transmission level and reported with a binomial 95% confidence interval.

The majority of low transmission settings are characterized by a high proportion of low-density infections, including areas with historically low transmission, such as Brazil [15,16], Haiti [17] and the Pacific islands [18]. However, a small number of settings with very low transmission (PCR prevalence below 1%) in Haiti [19], China [20], the Brazilian Amazon (Mueller, unpublished) and Solomon Islands [21] are exceptions to this general trend, as most *P. falciparum* infections were detectable by LM. It remains unknown whether after a prolonged period of sustained low-level transmission an inflection point is reached, after which most infections become detectable again by conventional diagnosis, or whether low-density infections that are undetectable by conventional diagnosis will persist. The small numbers of infected individuals per survey in low to very low transmission settings, and the large uncertainty of estimates associated with these small numbers, remain a problem for determining trends at this very low level of transmission. In these very low transmission settings, the choice of population at risk can further influence prevalence estimates, as the few positive cases may be found in small pockets or foci of transmission.

The proportion of infections detected by a diagnostic test further depends on the reference method used. Ultra-sensitive molecular methods, some of which assess high blood volumes [22], have uncovered a larger reservoir of low-density infections than anticipated by standard 18S rRNA qPCR both in a high-endemic area of Tanzania [23] and in low-endemic areas in South-East Asia [24,25]. Along the Thai–Myanmar border, the numerical distribution of parasite densities suggests that, even using ultra-sensitive molecular methods, about 25% of *P. falciparum* infections and 15% of *P. vivax* infections are missed [25].

Based on the parasite density distributions determined using NAA-based methods in the reviewed studies, and recognizing the poor comparability of parasite densities measured using different quantification methods across studies, on average 42% (range 1–97%) and 57% (range 11–100%) of all detectable infections were characterized by parasitaemia above 100 parasites/µl and 10 parasites/µl, respectively (Slater & Okell, meeting pre-read). In the reviewed studies, more than 80% (average of 89%) of *P. falciparum* infections were characterized by parasitaemia above 1 parasite/µl. The proportion of *P. falciparum* infections with low densities below 100 parasites/µl increased in low transmission settings relative to high transmission settings; however, no such trend was observed in the proportion of very-low-density *P. falciparum* infections below 10 parasites/µl.

4.1.4. The detectability of low-density P. falciparum and P. vivax infections in relation to the duration of the infection

Experimental malaria infections are characterized by an initial phase during which rising parasite densities are too low to be detected by conventional diagnostics. This phase is followed in most cases by an LM- or RDT-detectable peak in parasitaemia that often requires treatment. In experimental infections that are left untreated, or where only subcurative doses are applied to mitigate symptoms, the peak in parasitaemia is followed by chronic parasitaemia with fluctuating density, which eventually again falls to low densities undetectable by LM or RDT [26,27].

In data from experimental infections with *P. falciparum* and *P. vivax*, the mean time period between detection of infection by PCR and detection by LM at the start of an infection was approximately 3–5 days in non-immune individuals [28–30]. In a naturally exposed population in Western Kenya, a high transmission setting wherein individuals acquire semi-

immunity with repeated exposure, this period extended to 1 week in young children and to 3 weeks in adults [31].

Long-term persistence of low-level parasitaemia at the tail end of infections is considered more relevant for transmission than the shorter low-density phase at the beginning of the infection. Data from malaria therapy infections indicate a decreasing probability of detection by LM over the course of an infection for both species, but low-density periods (during which the infection is undetectable by LM) can occur early in infection. For *P. vivax*, data from malaria therapy infections have shown decreasing blood-stage densities and infection duration detected by LM with each relapse (i.e., non-primary period of parasitaemia) or homologous reinfection [27].

Data are scarce on the detectability of untreated *P. falciparum* and *P. vivax* infections throughout their duration using LM and/or NAA-based techniques in natural endemic settings. Studying the infection dynamics of natural malaria infections is complicated by superinfection and the interaction of concurrent clones in the host, as well as by host immunity that reduces parasite densities sometimes below the LOD of LM or even PCR [32,33]. For *P. vivax*, relapses contribute substantially to blood-stage infections [10] and add another layer of complexity to parasite detection patterns, even in the absence of superinfection. Using current parasite genotyping methods, it is not possible to distinguish between relapse and primary infection. Novel technologies such as amplicon sequencing [34] provide high-resolution genetic information and have the potential to measure clonal parasite densities, thus overcoming some of the limitations of current molecular methods used to investigate clonal infection dynamics. As such, these novel techniques may help to provide new insights into relapse–reinfection epidemiology and the course of natural infections.

Using statistical methods that take into account periods of low-density parasitaemia below the LOD of PCR, estimates of the mean duration of natural *P. falciparum* infections, per clone, range from several months (e.g., Ghana, 70–200 days [33]; Thailand, 135 days (White, unpublished)) to around 1 month (Papua New Guinea, 36 days (White, unpublished)). In studies in Vietnam (Nguyen, unpublished) and Thailand [35], 80–87% of participants remained *P. falciparum*-negative in monthly sampling after one initial detection by ultra-sensitive PCR. This suggests short infection durations (although the initial start times of the infection were unknown). By contrast, long persistence of some *P. falciparum* clones over several months was directly observed in Ghanaian infants [36] and over the dry season in Sudan [37,38]. In cohort studies in Africa and Papua New Guinea, low-density *P. falciparum* infections undetectable by LM were preceded and followed by PCR-negative samples in the majority of cases (Slater & Okell, meeting pre-read). In some of these cohorts, but not in others, the presence of such submicroscopic detections was a positive predictor for later LM detections (Slater & Okell, meeting pre-read).

Mathematical modelling suggests that individual *P. vivax* clones persist in the blood stream for 24–29 days in Thailand and Papua New Guinea, with relapses occurring every 41–55 days (White, unpublished). Although each individual blood-stage *P. vivax* infection seems shorter than those of *P. falciparum*, it is conceivable that *P. vivax* achieves comparable persistence through relapse. In Cambodia, untreated infections of *P. vivax* (detected by ultra-sensitive PCR) persisted over several months with densities fluctuating around the LOD of LM [35]. Preventing relapses through treatment with 8-aminoquinolines would effectively shorten *P. vivax* parasitaemia.

It cannot be determined from the majority of available data whether the longitudinally observed patterns in *P. falciparum* and *P. vivax* densities reflect ongoing infections, frequent superinfections due to high exposure, or relapse in the case of *P. vivax*. Across cohorts with data that were available and reviewed, the majority of participants that repeatedly carried submicroscopic *P. falciparum* or *P. vivax* infections became slide-positive at some point during follow-up. Only 0.8–18% of *P. falciparum* and 14–18% of *P. vivax* carriers remained submicroscopic throughout follow-up (Slater & Okell, Robinson, meeting pre-reads).

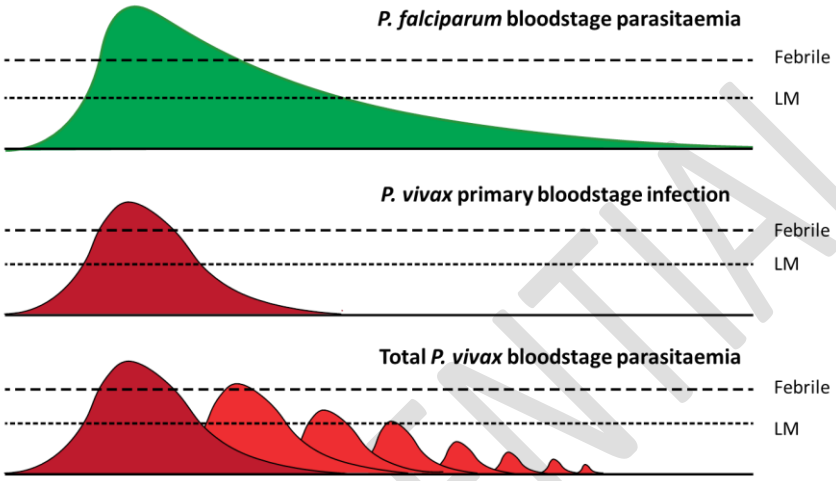

Figure 2. Models of the average pattern of *P. falciparum* and *P. vivax* blood-stage infection dynamics. Blood-stage parasitaemia is depicted in the absence of superinfections. Within individual infections, there are fluctuations in density. Figure taken from the ERG presentation by Ivo Mueller.

4.1.5. The duration of infectiousness in P. falciparum and P. vivax in treated infections

In accordance with their different infection dynamics, schizonticidal antimalarial treatment (i.e., acting only on the asexual blood stages) influences infectiousness in different ways for *P. falciparum* and *P. vivax*. Treatment of a *P. falciparum* infection truncates the infection and interrupts further generation of new gametocytes that contribute to transmission events. Residual circulating mature *P. falciparum* gametocytes maintain transmission in the short-term after treatment, with the duration of gametocyte persistence dependent on the type of (artemisinin combination) therapy used [39]. Primaquine (PQ) treatment reduces the risk of post-treatment transmission of the infection [40]. Treatment of *P. vivax* infections using only blood-stage-clearing drugs does not have an effect against transmission events occurring from subsequent relapses. Only clearance of liver hypnozoites can abrogate future infectivity of *P. vivax* carriers [41,42].

Key conclusions

- The terms “submicroscopic”, “asymptomatic” and “low-density” infection are often used interchangeably in the published literature. Harmonization of terminology and definitions should be promoted. The ERG agreed that “low-density infection” is the preferred term and it should be defined in each publication by stating the applied parasitaemia cut-off (suggested: <100 parasites/µl) along with the molecular method used for quantification. In studies that do not quantify parasitaemia, low-density infections can be defined as those identified through highly sensitive methods but not through conventional diagnostics. Similarly, the use of the term “asymptomatic” should be defined in terms of the specific symptoms recorded and the time period evaluated in relation to infection detection.
- Standardized reporting and harmonization of molecular methods is needed to ensure accuracy of results and comparability between studies. In particular, the LOD of the NAA method (determined using quality-assured reference materials such as the WHO international DNA standard for *P. falciparum*) and the equivalent of blood volume added to the NAA reaction should be specified. A detailed description of the molecular workflow should consist of the specification of the sample type, including the volume sampled, storage conditions, extraction method, amplification method and identifier of target sequence.
- Approximately half of *P. falciparum* and *P. vivax* infections detected in cross-sectional surveys were detected by conventional diagnostics. The proportion of infected individuals with low-density malaria infections detected by more sensitive tests increased in low transmission settings, although absolute numbers of carriers were higher in high transmission settings. There are limited data and high uncertainty with regard to estimates in very low transmission settings. More studies are required that also consider recent history of transmission and the potential impact of residual immunity in the population.
- There are indications that new infections are more likely to be detected by conventional diagnostics than chronic infections, which tend to have lower parasite densities. Infection dynamics of natural infections remain understudied due to limitations of the current molecular genotyping methods and the complexity of required study designs. A better understanding of the longitudinal dynamics and detectability of infections is relevant, particularly in low-endemic settings where superinfections are rare.

4.2 The contribution to transmission of low-density *P. falciparum* and *P. vivax* infections

4.2.1. Factors influencing the likelihood of P. falciparum *and* P. vivax *transmission to mosquitoes*

The wider use of molecular methods to detect and quantify *P. falciparum* and *P. vivax* gametocytes in epidemiological surveys, complemented by experimental mosquito feeding studies, has generated evidence to evaluate the infectiousness to mosquitoes of various parasite and gametocyte densities.

For *P. vivax,* changes in gametocyte densities closely follow those of asexual stages. A variety of studies have shown a clear correlation between *P. vivax* gametocytaemia and total parasitaemia, including in the low-density range, with an asexual parasite to gametocyte ratio of 10:1 [43–45]. The presence and density of *P. falciparum* gametocytes are less well-correlated with asexual parasitaemia because of the long gametocyte maturation and circulation time, and sequestration. Therefore, no clear relationship exists between asexual density and concurrent gametocyte density for *P. falciparum* [44], although a trend of lower *P. falciparum* gametocyte densities has been observed in low-density infections compared to LM-detectable infections (Slater & Okell, meeting pre-read).

Because of their better reproducibility and standardization, as well as due to ethical considerations, membrane feeding assays (MFAs) are commonly used in epidemiological studies rather than direct feeding on skin. However, MFAs do not capture all the elements of mosquitoes' natural skin feeding that might influence transmission. A variety of vector, host and parasite factors further influence the outcome of mosquito feeding experiments. These include (but are not limited to) (i) vector species, density and age; (ii) host immunity, age and symptomatic status; and (iii) parasite and gametocyte density. Few studies have investigated the relevance and individual impact of each of these factors [46], which remain poorly understood and are setting-dependent.

For *P. falciparum* and *P. vivax*, the likelihood of mosquito infection in experimental feeding experiments increases with increases in gametocyte density, exhibiting an S-shaped dose-response relationship; at very low gametocyte density, there is a low likelihood of mosquito infection, whereas above gametocyte densities of 200 to 1000 gametocytes/µl, there is saturation without further increase in the prevalence of infected mosquitoes [43,47–49]. For *P. vivax*, studies in Thailand [49] and Ethiopia (Tadesse, unpublished) described a steep increase in the probability of mosquito infection at a parasite density of approximately 10 parasites/µl. This level coincides with the LOD of expert microscopy. These studies showed that low-density *P. vivax* infections below this threshold were unlikely to transmit to mosquitoes, whereas LM-detectable infections frequently transmitted to mosquitoes, generating high infection rates. As a result, field microscopy with an LOD of around 100 parasites/µl may miss *P. vivax* densities that are readily infectious to mosquitoes. For *P. falciparum,* successful transmission events have frequently been observed at low parasite or low gametocyte densities, but the variation in infectivity data is high [43,47,48].

The available evidence does not facilitate the evaluation of the influence of vector species, parasite strains, epidemiological settings and the host's symptomatic status on the relationship between parasitaemia or gametocyte densities and the likelihood of transmission to mosquitoes. Current experimental systems are limited to investigating human-to-mosquito transmission, but cannot provide information about the likelihood of subsequent mosquito-to-human transmission. Although there are few data on the probability of host infection after exposure to mosquitoes with varying sporozoite loads in the salivary gland, available data indicate a saturating relationship in both *P. berghei* and *P. falciparum* [50]. While the current evidence suggests that even single oocyst infections in *P. falciparum* give rise to hundreds or thousands of sporozoites in the salivary glands [51], and recognizing that oocyst densities in the majority of wild-caught *Anopheles* range between one and three oocysts [52], understanding the likelihood of secondary infections from mosquitoes with a low infection burden is of importance for understanding the relevance of low parasite densities to malaria transmission.

The natural history of infection and longitudinal infection dynamics need to be considered in evaluating the transmission potential of natural infections. While infectiousness was extensively studied in early malaria therapy studies, in all more recent studies, it has been assessed at one point in time without accounting for the dynamic nature of malaria blood-stage infections characterized by oscillating density. Longitudinal studies of the infectivity dynamics of natural infections are lacking.

4.2.2. The P. falciparum *and* P. vivax *low-density infectious reservoir*

To estimate the contribution to transmission of the low-density *P. falciparum* infectious reservoir, i.e., the combined infectivity of a population to mosquitoes [53], data were available for review from five recent studies in high transmission areas in Burkina Faso, Kenya and Senegal, with slide prevalence ranging from 26% to 49% (Goncalves, unpublished; [54,55]). In these studies, 32–65% of *P. falciparum* infections detected by NAA-based methods were not detectable by LM; these contributed an estimated 15–30% of mosquito infections (Slater & Okell, meeting pre-read). In a re-analysis of data from a study in Kenya with 84% slide prevalence [56], *P. falciparum* infections not detectable by LM were rare and only contributed an estimated 2.3% of mosquito infections. In these six studies the proportion of the infectious reservoir not detected by LM but detected using diagnostics with different LODs increased by 10–30% using a diagnostic with an LOD of 100 parasites/µl and by 70–80% using a highly sensitive diagnostic with an LOD of 1 parasite/µl. Including the LM-detectable infectious reservoir, it is estimated that a highly sensitive diagnostic with an LOD of 1 parasite/µl would detect 83–96% of the total infectious reservoir.

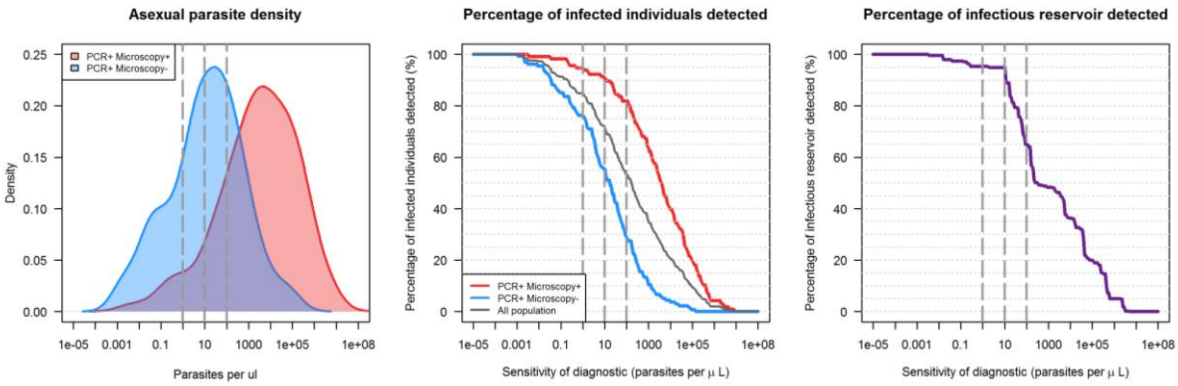

Figure 3. Parasite density distributions, determined using quantitative NAA methods, for individuals from Burkina Faso that are detectable (red) and undetectable (blue) by microscopy (left panel); and the proportion of infected individuals (middle panel) and of the infectious reservoir (right panel) detected at different diagnostic sensitivity thresholds (1, 10 and 100 parasites/µl). Figure from Slater & Okell, meeting pre-read, unpublished data.

The contribution of the low-density infectious reservoir to maintaining malaria transmission is estimated to be higher in areas of low or moderate parasite prevalence than in areas of high parasite prevalence [5], and depends on a variety of vectorial and environmental factors as well as population immunity. Vector species, vectorial capacity and local environmental factors, such as the presence/absence of mosquito–human contact sites, quality of health systems and rate of treatment of infections, can impact the likelihood of onward transmission of low-density infections to mosquitoes. It is currently unclear as to which of these factors are most relevant for maintaining transmission in different endemic

settings, and which are particularly relevant or limiting for the transmission of low-density infections. Most infectivity studies to date have been performed in high transmission settings and comparable studies are lacking in low or moderate transmission settings. In low transmission settings, the screening of large populations is required in order to identify parasite carriers. This is an expensive and labour-intensive task. In addition, quality control for feeding procedures is more complex in low-endemic areas. Quality control of transmission studies requires the recruitment of high-density gametocyte carriers (for which the proportion of infected mosquitoes that can be expected is reasonably well described); however, these high-density gametocyte carriers are less common in low-endemic settings compared to moderate- and high-endemic settings. Infectivity studies may therefore be more feasible in moderate transmission settings than in low transmission settings.

Key conclusions

- For both *P. falciparum* and *P. vivax*, the likelihood and intensity of mosquito infection is positively, but not linearly, associated with gametocyte density. Transmissibility to malaria vectors is less efficient at very low gametocyte densities and plateaus above certain high levels of gametocyte density. The host, parasite and vectorial factors that modify this relationship are not well understood. Critical data are lacking on the likelihood of subsequent mosquito-to-human transmission, and the relationship between sporozoite load and the probability of human infection in natural infections of the human malarias.

- For *P. falciparum*, but less so for *P. vivax*, transmission events occur regularly at low parasite densities below the LOD of expert microscopy (estimated at 10 parasites/µl). At the LOD of non-expert or field microscopy, *P. vivax* parasite densities that are readily infectious to mosquitoes can be missed. There is no evidence of a measurable parasite density threshold below which transmission cannot occur.

- Evidence from a limited number of areas with a high prevalence of *P. falciparum* indicates that low-density infections can be responsible for more than 15% of mosquito infections in these settings. Evidence is lacking from areas with low *P. falciparum* prevalence and for *P. vivax*.

- Current evidence is insufficient for understanding the contribution of low-density *P. falciparum* or *P. vivax* infections to onward transmission to human populations. Intervention trials to directly assess the effect of identifying and treating low-density infections are warranted.

4.3 Relevance of detecting low-density P. falciparum and P. vivax infections

Based on the available evidence, the ERG participants discussed the relevance of detecting low-density *P. falciparum* and *P. vivax* infections in different endemic settings as part of research activities, surveillance or intervention strategies.

It was agreed that the detection of low-density infections has no current role in the case management of suspected malaria clinical cases or in the surveillance of clinical malaria

cases. Currently, there is no evidence to support the use of a highly sensitive HRP2-based POC test for the diagnosis of clinical malaria or surveillance. Conventional RDTs and quality LM are sufficiently sensitive to detect *P. falciparum* densities most commonly associated with the signs and symptoms of clinical malaria.

Considering that in low transmission settings (including those targeted for elimination) low-density infections account for a high proportion of the total number of infections in cross-sectional surveys, and as there is currently no known measurable parasite density threshold below which transmission cannot occur, research is needed to explore the potential impact and cost–effectiveness of highly sensitive POC tests, such as a highly sensitive RDT in active or reactive case detection strategies, for reducing transmission.

Research should target scenarios in which the detection of low-density infections may be most relevant: epidemiological field trials to measure the impact of identifying and treating all infections, including low-density infections, on transmission, border-screening of immigrants or migrant populations, and foci mapping and investigations. Other potential scenarios may include the screening and treatment of pregnant women in antenatal care in order to study how low or sequestered parasites undetectable by conventional diagnostics impact pregnancy outcomes. The potential benefits of detecting and monitoring low-density infections may be specifically investigated in areas where antimalarial resistance occurs, in order to assess the dynamics of natural infections in relation to changes of resistance markers and their role in the development and spread of resistance.

Intervention strategies targeting low-density infections are not applicable in high-transmission settings and carry an increased risk of significant resources being diverted away from clinical case management and conventional diagnostic tests, which are more cost–effective in these settings.

Key conclusions

- Highly sensitive tests have no proven benefit over conventional diagnostics in routine malaria case management and the surveillance of clinical cases.
- Research is needed to document the public health benefits and cost–effectiveness of detecting and treating low-density infections in low transmission areas and/or specific population groups. In particular, potential research objectives for highly sensitive diagnostic tests may include: epidemiological research to measure the impact of identifying and treating all infections, including low-density infections, on transmission, border-screening of immigrants or migrant populations, foci mapping and investigations in the context of malaria elimination, and the detection and treatment of low or sequestered parasite biomass in pregnant women.

4.4 Clinical management and surveillance of low-density *P. falciparum* and *P. vivax* infections

Discussions focused on individual versus community risks and the benefits of treating low-density asymptomatic infections. Ethical considerations with respect to the need for follow-

up or treatment of low-density asymptomatic infections in research settings were also discussed.

4.4.1. Clinical consequences of low-density P. falciparum and P. vivax infections

Persistent asymptomatic malaria infections can contribute to a range of clinical consequences, including (but not limited to) repeated acute illness episodes, all-cause morbidity and mortality (indicated by an excess reduction of morbidity and mortality due to malaria control interventions), malaria-related anaemia, splenomegaly, placental malaria with consequences for both mother and infant, coinfection with invasive bacterial disease, and cognitive impairment [57].

The ERG participants agreed that the current evidence is not sufficient to evaluate the clinical consequences of low-density asymptomatic infections with respect to the natural history of the individual infection. Low-density infections may represent chronic, self-resolving, or pre-recrudescent infections. Currently, it is not known whether a detected low-density infection is a marker of previous or future symptomatic malaria, or a marker of previous exposures to malaria and thus cumulative immunity, and how this relationship changes in different endemic settings.

Asymptomatic low-density infections may be important for maintaining clinical immunity in the presence of ongoing exposure. A recent study in Mali found that, for children initially carrying a chronic asymptomatic *P. falciparum* infection, the risk of clinical malaria was reduced over two transmission seasons compared to children without a diagnosed malaria infection. This reduction in risk was comparable for children in whom the chronic infection was treated (RDT-positive children) and for children in whom infection was allowed to persist (RDT-negative, PCR-positive) [58]. In one recent study in Zambia, asymptomatic and symptomatic malaria infections appeared to be associated with genetically distinct parasite subpopulations [59]. More studies are required to evaluate the relevance of chronic low-density malaria infections for maintaining clinical immunity in different endemic settings.

4.4.2. Treatment of low-density P. falciparum and P. vivax infections in research versus programmatic settings

There is a large body of evidence supporting the negative clinical consequences of asymptomatic *P. falciparum* and *P. vivax* infections [57], as well as the role of submicroscopic malaria infections in defining the human infectious reservoir for *P. falciparum* malaria [5]. There is, however, limited evidence on the prospective clinical and pathological impact of asymptomatic low-density infections that are undetectable by conventional diagnostics. In programmatic settings, the risks and benefits of treating asymptomatic, low-density infections have to be weighed at both the individual and the community level.

At the individual level, every malaria infection detected, irrespective of parasite density, should receive appropriate treatment to prevent future morbidity and mortality. The ERG agreed that at the individual level this benefit outweighs the risks associated with treating the infection; such risks may be related to drug adverse effects or the loss of the potential protective effect of chronic infection against clinical malaria. However, given the current state of knowledge, the added cost of seeking, finding and treating low-density malaria infections (detected in asymptomatic individuals or patients presenting with fever of non-malarial origin) should not divert resources away from the management of symptomatic

malaria cases and other components of national malaria control programmes. Studies to determine the impact and cost–effectiveness of treating low-density infections in routine clinical practice or surveillance are essential for guiding the use (and subsequent treatment actions) of highly sensitive POC diagnostics in high to medium transmission settings.

In research settings, case management and treatment of low-density malaria infections should generally be provided according to the research protocol approved by the national ethics review committee. In research settings, infections are frequently not detected at the POC but retrospectively, making it operationally challenging to trace the participants and treatment. Where the aim of the research activity consists of monitoring longitudinal aspects of infections, provision of antimalarial treatment at enrolment or during follow-up may interfere directly with the research aim. Given that there is limited evidence to indicate that low-density infections are associated with significant future malaria morbidity, treatment of asymptomatic infections identified at study contact in research settings may be withheld after consultation with national ethics review committees, provided that positive participants' signs and symptoms of malaria can be closely monitored. Appropriate care should be given to infected individuals presenting with symptoms. If infections are identified retrospectively, every effort should be made to raise awareness in the study area on the risks and symptoms of malaria infection and encourage appropriate care-seeking behaviour.

4.4.3. Reporting of low-density P. falciparum *and* P. vivax *infections in the surveillance system*

The availability and use of a highly sensitive RDT as part of active or reactive case detection in malaria programmes is likely to result in the increased detection and reporting of low-density parasitaemias. The ERG agreed that malaria cases found through active or reactive case detection should be reported separately from those detected passively, preferably along with the mode of diagnosis and the denominator of the population screened. When reporting, the diagnostic method used should be indicated (e.g., conventional RDTs, LM, highly sensitive RDTs or specific NAA-based methods). In addition, the type of diagnostic used should be taken into account in trend analysis, intervention targeting and impact evaluation. The comparability of measures between years is crucial for trend analysis.

Further research is required to identify the most cost–effective deployment strategy of highly sensitive diagnostics for malaria surveillance. A better understanding of the proportion of low-density infections that need to be identified and treated in order to reduce transmission in different transmission and epidemiological settings is crucial for designing cost–effective implementation strategies. Controlled trials of active and reactive case detection (such as FSAT or MSAT using highly sensitive diagnostics) compared to relevant interventions (such as MDA, reactive case detection using conventional diagnostics, or universal access to diagnosis and treatment and vector control) are required to assess the potential role of highly sensitive diagnostics in accelerating malaria elimination. These trials will generate the evidence to inform future WHO recommendations on detection schemes of low-density infections for malaria elimination and certification of malaria-free status.

Key conclusions

- A significant proportion of asymptomatic infections are characterized by parasite density that is below the LOD of LM or conventional RDT. Low-density infections are frequently detected also in febrile patients, particularly in high-endemic areas, but these may not be the underlying cause of fever. The available evidence is not sufficient to fully evaluate the prospective clinical and pathological impact of untreated low-density malaria infections.
- In programmatic settings, every detected malaria case (including low-density malaria infections) should receive appropriate treatment. Appropriate treatment should include PQ for P. vivax cases.
- In research settings, appropriate care should be given to infected individuals in line with national ethics committee requirements. In research scenarios in which low-density infections are identified retrospectively or in which treatment would directly interfere with the study aim, treatment of asymptomatic malaria cases may occasionally be withheld only if close monitoring for signs and symptoms of acute malaria is provided.
- Malaria cases identified by active or reactive case detection should be reported separately from those detected passively, along with the mode of diagnosis and the denominator of the population screened.
- Controlled trials of active and reactive case detection using RDTs and highly sensitive RDTs are required in low transmission settings in order to assess the impact on transmission of detecting and treating low-density asymptomatic infections, and to design cost–effective strategies for their use by malaria programmes.

4.5. Priority research questions

There is a need to better understand the contribution of low-density infections to transmission to human populations in endemic communities, and to directly evaluate the impact on transmission by actively detecting and treating low-density infections in intervention trials in different endemic and epidemiological settings.

Specific research questions:

- What is the proportion and absolute number of low-density infections in low and very low transmission settings (0–5% prevalence by PCR), and what is the spatial distribution of malaria infections?
- What is the relationship between the proportion of low-density infections and recent history of transmission, i.e., is an inflection point reached in the proportion of low-density infections detected by highly sensitive diagnostics in areas with sustained reduction of transmission at very low levels?
- What is the proportion of low-density asymptomatic infections that become symptomatic as part of the natural history of infection in different endemic settings?

- What is the prospective clinical and pathological impact of untreated low-density parasitaemia?
- What are the risk factors for persistence, and what is the role of low-density infections in the spread of antimalarial resistance?
- Can novel molecular techniques such as amplicon sequencing aid in investigating the natural history of infections, e.g., by measuring clonal parasite density, and in investigating relapse–reinfection epidemiology?
- In the natural history of infections, what is the duration of infectiousness (particularly in low-endemic settings) and what are its major determinants?
- What are the main determinants – related to host, vector and parasite – of infection success in experimental mosquito-feeding experiments and of making those mosquitoes infectious for humans? What is the relationship between parasite density and infectiousness for different vector species? What are feasible study designs with which to achieve meaningful numbers in low-endemic settings?

The participants agreed that many of the research questions listed above are unlikely to be answered within the timeframe required to form an evidence base for guiding malaria control programmes and elimination strategies.

Immediate programmatic open research questions are:

- What impact on transmission is achievable by actively detecting and eliminating all infections, including low-density malaria infections, using highly sensitive POC diagnostics in low transmission settings, particularly in areas of low vectorial capacity, compared to conventional malaria elimination methods (i.e., universal access to diagnosis and treatment and vector control), MDA, and active or reactive screen-and-treat campaigns using less sensitive POC diagnostics?
- In low and very low transmission settings, what is the proportion (or number) of infections that need to be detected and treated in order to accelerate the reduction of transmission towards malaria elimination?
- What is the cost–benefit for health systems in using highly sensitive diagnostics for specific target groups and in elimination settings? What are the most cost–effective deployment strategies for highly sensitive diagnostics in different settings?

References

1. WHO policy recommendation on malaria diagnostics in low transmission settings. Geneva: World Health Organization; 2014 (http://www.who.int/malaria/publications/atoz/diagnotics-low-transmission-settings/en/, accessed 18 May 2017).

2. Snounou G, Viriyakosol S, Jarra W, Thaithong S, Brown KN. Identification of the four human malaria parasite species in field samples by the polymerase chain reaction and detection of a high prevalence of mixed infections. Mol. Biochem. Parasitol. 1993;58:283–92.

3. Okell LC, Ghani AC, Lyons E, Drakeley CJ. Submicroscopic infection in Plasmodium falciparum-endemic populations: a systematic review and meta-analysis. J. Infect. Dis. 2009;200:1509–17.

4. Cheng Q, Cunningham J, Gatton ML. Systematic review of sub-microscopic P. vivax infections: prevalence and determining factors. PLoS Negl. Trop. Dis. 2015;9:e3413.

5. Okell LC, Bousema T, Griffin JT, Ouédraogo AL, Ghani AC, Drakeley CJ. Factors determining the occurrence of submicroscopic malaria infections and their relevance for control. Nat. Commun. 2012;3:1237.

6. Das S, Jang IK, Barney B, Peck R, Rek JC, Arinaitwe E, et al. Performance of a high-sensitivity rapid diagnostic test for Plasmodium falciparum malaria in asymptomatic individuals from Uganda and Myanmar and naive human challenge infections. Am. J. Trop. Med. Hyg. 2017. doi: 10.4269/ajtmh.17-0245.

7. Imwong M, Stepniewska K, Tripura R, Peto TJ, Lwin KM, Vihokhern B, et al. Numerical distributions of parasite densities during asymptomatic malaria. J. Infect. Dis. 2015;213:1322–9.

8. Bustin SA, Benes V, Garson JA, Hellemans J, Huggett J, Kubista M, et al. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. Clin. Chem. 2009;55:611–22.

9. Lin JT, Saunders DL, Meshnick SR. The role of submicroscopic parasitemia in malaria transmission: what is the evidence? Trends Parasitol. 2014;30:183–90.

10. Robinson LJ, Wampfler R, Betuela I, Karl S, White MT, Li Wai Suen CSN, et al. Strategies for understanding and reducing the Plasmodium vivax and Plasmodium ovale hypnozoite reservoir in Papua New Guinean children: a randomised placebo-controlled trial and mathematical model. PLoS Med. 2015;12:e1001891.

11. Morris U, Xu W, Msellem MI, Schwartz A, Abass A, Shakely D, et al. Characterising temporal trends in asymptomatic Plasmodium infections and transporter polymorphisms during transition from high to low transmission in Zanzibar, 2005–2013. Infect. Genet. Evol. 2015;33:110–7.

12. Laban NM, Kobayashi T, Hamapumbu H, Sullivan D, Mharakurwa S, Thuma PE, et al. Comparison of a PfHRP2-based rapid diagnostic test and PCR for malaria in a low prevalence setting in rural southern Zambia: implications for elimination. Malar. J. 2015;14:25.

13. Kangoye DT, Noor A, Midega J, Mwongeli J, Mkabili D, Mogeni P, et al. Malaria hotspots defined by clinical malaria, asymptomatic carriage, PCR and vector numbers in a low transmission area on the Kenyan Coast. Malar. J. 2016;15:213.

14. Shekalaghe SA, Drakeley C, van den Bosch S, ter Braak R, van den Bijllaardt W, Mwanziva C, et al. A cluster-randomized trial of mass drug administration with a gametocytocidal drug combination to interrupt malaria transmission in a low endemic area in Tanzania. Malar. J. 2011;10:247.

15. da Silva NS, da Silva-Nunes M, Malafronte RS, Menezes MJ, D'Arcadia RR, Komatsu NT, et al. Epidemiology and control of frontier malaria in Brazil: lessons from community-based studies in rural Amazonia. Trans. R. Soc. Trop. Med. Hyg. 2010;104:343–50.

16. Ladeia-Andrade S, Ferreira MU, de Carvalho ME, Curado I, Coura JR. Age-dependent acquisition of protective immunity to malaria in riverine populations of the Amazon Basin of Brazil. Am. J. Trop. Med. Hyg. 2009;80:452–9.

17. Eisele TP, Keating J, Bennett A, Londono B, Johnson D, Lafontant C, et al. Prevalence of Plasmodium falciparum infection in rainy season, Artibonite Valley, Haiti, 2006. Emerg. Infect. Dis. 2007;13:1494–6.

18. Pacific Malaria Initiative Survey Group (PMISG) on behalf of the Ministries of Health of Vanuatu and Solomon Islands. Malaria on isolated Melanesian islands prior to the initiation of malaria elimination activities. Malar. J. 2010;9:218.

19. Lucchi NW, Karell MA, Journel I, Rogier E, Goldman I, Ljolje D, et al. PET-PCR method for the molecular detection of malaria parasites in a national malaria surveillance study in Haiti, 2011. Malar. J. 2014;13:462.

20. Cheng Z, Wang D, Tian X, Sun Y, Sun X, Xiao N, et al. Capture and ligation probe-PCR (CLIP-PCR) for molecular screening, with application to active malaria surveillance for elimination. Clin. Chem. 2015;61:821–8.

21. Waltmann A, Darcy AW, Harris I, Koepfli C, Lodo J, Vahi V, et al. High rates of asymptomatic, sub-microscopic Plasmodium vivax infection and disappearing Plasmodium falciparum malaria in an area of low transmission in Solomon Islands. PLoS Negl. Trop. Dis. 2015;9:e0003758.

22. Imwong M, Hanchana S, Malleret B, Rénia L, Day NPJ, Dondorp A, et al. High-throughput ultrasensitive molecular techniques for quantifying low density malaria parasitemias. J. Clin. Microbiol. 2014;52:3303–9.

23. 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.

24. Imwong M, Nguyen TN, Tripura R, Peto TJ, Lee SJ, Lwin KM, et al. The epidemiology of subclinical malaria infections in South-East Asia: findings from cross-sectional surveys in Thailand–Myanmar border areas, Cambodia, and Vietnam. Malar. J. 2015;14:381.

25. Imwong M, Stepniewska K, Tripura R, Peto TJ, Lwin KM, Vihokhern B, et al. Numerical distributions of parasite densities during asymptomatic malaria. J. Infect. Dis. 2016;213:1322–9.

26. Collins WE, Jeffery GM. A retrospective examination of sporozoite- and trophozoite-induced infections with Plasmodium falciparum in patients previously infected with heterologous species of Plasmodium: effect on development of parasitologic and clinical immunity. Am. J. Trop. Med. Hyg. 1999;61:36–43.

27. Collins WE, Jeffery GM, Roberts JM. A retrospective examination of reinfection of humans with Plasmodium vivax. Am. J. Trop. Med. Hyg. 2004;70:642–4.

28. Murphy SC, Prentice JL, Williamson K, Wallis CK, Fang FC, Fried M, et al. Real-time quantitative reverse transcription PCR for monitoring of blood-stage Plasmodium falciparum infections in malaria human challenge trials. Am. J. Trop. Med. Hyg. 2012;86:383–94.

29. Lyke KE, Laurens M, Adams M, Billingsley PF, Richman A, Loyevsky M, et al. Plasmodium falciparum malaria challenge by the bite of aseptic Anopheles stephensi mosquitoes: results of a randomized infectivity trial. PLoS One. 2010;5:e13490.

30. Payne RO, Griffin PM, McCarthy JS, Draper SJ. Plasmodium vivax controlled human malaria infection: progress and prospects. Trends Parasitol. 2017;33:141–50.

31. Pinkevych M, Petravic J, Chelimo K, Vulule J, Kazura JW, Moormann AM, et al. Decreased growth rate of P. falciparum blood stage parasitemia with age in a holoendemic population. J. Infect. Dis. 2014;209:1136–43.

32. Bretscher MT, Valsangiacomo F, Owusu-Agyei S, Penny MA, Felger I, Smith T. Detectability of Plasmodium falciparum clones. Malar. J. 2010;9:234.

33. Felger I, Maire M, Bretscher MT, Falk N, Tiaden A, Sama W, et al. The dynamics of natural Plasmodium falciparum infections. PLoS One. 2012;7:e45542.

34. Lin JT, Hathaway NJ, Saunders DL, Lon C, Balasubramanian S, Kharabora O, et al. Using amplicon deep sequencing to detect genetic signatures of Plasmodium vivax relapse. J. Infect. Dis. 2015;212:999–1008.

35. Tripura R, Peto TJ, Chalk J, Lee SJ, Sirithiranont P, Nguon C, et al. Persistent Plasmodium falciparum and Plasmodium vivax infections in a western Cambodian population: implications for prevention, treatment and elimination strategies. Malar. J. 2016;15:181.

36. Franks S, Koram KA, Wagner GE, Tetteh K, McGuinness D, Wheeler JG, et al. Frequent and persistent, asymptomatic Plasmodium falciparum infections in African infants, characterized by multilocus genotyping. J. Infect. Dis. 2001;183:796–804.

37. Babiker HA. Unstable malaria in Sudan: the influence of the dry season. Plasmodium falciparum population in the unstable malaria area of eastern Sudan is stable and genetically complex. Trans. R. Soc. Trop. Med. Hyg. 1998;92:585–9.

38. Babiker HA, Abdel-Muhsin AA, Hamad A, Mackinnon MJ, Hill WG, Walliker D. Population dynamics of Plasmodium falciparum in an unstable malaria area of eastern Sudan. Parasitology. 2000;120 (Pt 2):105–11.

39. WWARN Gametocyte Study Group. Gametocyte carriage in uncomplicated Plasmodium falciparum malaria following treatment with artemisinin combination therapy: a systematic review and meta-analysis of individual patient data. BMC Med. 2016;14:79.

40. Graves PM, Gelband H, Garner P. Primaquine or other 8-aminoquinoline for reducing Plasmodium falciparum transmission. Cochrane Database Syst. Rev. 2015;2:CD008152.

41. Douglas NM, Simpson JA, Phyo AP, Siswantoro H, Hasugian AR, Kenangalem E, et al. Gametocyte dynamics and the role of drugs in reducing the transmission potential of Plasmodium vivax. J. Infect. Dis. 2013;208:801–12.

42. White M, Amino R, Mueller I. Theoretical implications of a pre-erythrocytic Plasmodium vivax vaccine for preventing relapses. Trends Parasitol. 2017;33:260–3.

43. Bousema T, Drakeley C. Epidemiology and infectivity of Plasmodium falciparum and Plasmodium vivax gametocytes in relation to malaria control and elimination. Clin. Microbiol. Rev. 2011;24:377–410.

44. Koepfli C, Robinson LJ, Rarau P, Salib M, Sambale N, Wampfler R, et al. Blood-stage parasitaemia and age determine Plasmodium falciparum and P. vivax gametocytaemia in Papua New Guinea. PLoS One. 2015;10:e0126747.

45. Douglas NM, Simpson JA, Phyo AP, Siswantoro H, Hasugian AR, Kenangalem E, et al. Gametocyte dynamics and the role of drugs in reducing the transmission potential of Plasmodium vivax. J. Infect. Dis. 2013;208:801–12.

46. Vallejo AF, Rubiano K, Amado A, Krystosik AR, Herrera S, Arévalo-Herrera M. Optimization of a membrane feeding assay for Plasmodium vivax infection in Anopheles albimanus. PLoS Negl. Trop. Dis. 2016;10:e0004807.

47. Johnston GL, Smith DL, Fidock DA. Malaria's missing number: calculating the human component of R0 by a within-host mechanistic model of Plasmodium falciparum infection and transmission. PLoS Comput. Biol. 2013;9:e1003025.

48. Da DF, Churcher TS, Yerbanga RS, Yaméogo B, Sangaré I, Ouedraogo JB, et al. Experimental study of the relationship between Plasmodium gametocyte density and infection success in mosquitoes: implications for the evaluation of malaria transmission-reducing interventions. Exp. Parasitol. 2015;149:74–83.

49. Kiattibutr K, Roobsoong W, Sriwichai P, Saeseu T, Rachaphaew N, Suansomjit C, et al. Infectivity of symptomatic and asymptomatic Plasmodium vivax infections to a Southeast Asian vector, Anopheles dirus. Int. J. Parasitol. 2017;47:163–70.

50. Churcher TS, Sinden RE, Edwards NJ, Poulton ID, Rampling TW, Brock PM, et al. Probability of transmission of malaria from mosquito to human is regulated by mosquito parasite density in naïve and vaccinated hosts. PLoS Pathog. 2017;13:e1006108.

51. Stone WJR, Eldering M, van Gemert G-J, Lanke KHW, Grignard L, van de Vegte-Bolmer MG, et al. The relevance and applicability of oocyst prevalence as a read-out for mosquito feeding assays. Sci. Rep. 2013;3:3418.

52. Saul A. Efficacy model for mosquito stage transmission blocking vaccines for malaria. Parasitology. 2008;135:1497–506.

53. Stone W, Gonçalves BP, Bousema T, Drakeley C. Assessing the infectious reservoir of falciparum malaria: past and future. Trends Parasitol. 2015;31:287–96.

54. Lin Ouédraogo A, Gonçalves BP, Gnémé A, Wenger EA, Guelbeogo MW, Ouédraogo 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.

55. Gaye A, Bousema T, Libasse G, Ndiath MO, Konaté L, Jawara M, et al. Infectiousness of the human population to Anopheles arabiensis by direct skin feeding in an area hypoendemic for malaria in Senegal. Am. J. Trop. Med. Hyg. 2015;92:648–52.

56. Githeko AK, Brandling-Bennett AD, Beier M, Atieli F, Owaga M, Collins FH. The reservoir of Plasmodium falciparum malaria in a holoendemic area of western Kenya. Trans. R. Soc. Trop. Med. Hyg. 1992;86:355–8.

57. Chen I, Clarke SE, Gosling R, Hamainza B, Killeen G, Magill A, et al. "Asymptomatic" malaria: a chronic and debilitating infection that should be treated. PLoS Med. 2016;13:e1001942.

58. Portugal S, Tran TM, Ongoiba A, Bathily A, Li S, Doumbo S, et al. Treatment of chronic asymptomatic Plasmodium falciparum infection does not increase the risk of clinical malaria upon reinfection. Clin. Infect. Dis. 2017;64:645–53.

59. Searle KM, Katowa B, Kobayashi T, Siame MNS, Mharakurwa S, Carpi G, et al. Distinct parasite populations infect individuals identified through passive and active case detection in a region of declining malaria transmission in southern Zambia. Malar. J. 2017;16:154.

Annex 1: Outcome of the working groups and review by meeting participants

The working groups discussed several suggestions for updating the current WHO recommendation on the diagnosis of *P. falciparum* and *P. vivax* malaria in low transmission settings and the proposed changes are listed below.

Recommendation 1 (current wording):

Quality assured RDT and microscopy are the primary diagnostic tools for the confirmation and management of suspected clinical malaria in all epidemiological situations, including areas of low transmission, due to their high diagnostic performance in detecting clinical malaria, their wide availability and relatively low cost. Similarly, RDT and microscopy are appropriate tools for routine malaria surveillance (of clinical cases) in the majority of malaria-endemic settings.

Suggested changes:

- […] Similarly, conventional RDT and microscopy are appropriate tools for routine malaria surveillance (of clinical cases) in the majority of malaria-endemic settings. Malaria cases should be reported by type of diagnostic test used.

Recommendation 2 (current wording):

A number of nucleic acid amplification techniques are available and are more sensitive in detection of malaria compared to RDTs and microscopy. Generally, the use of more sensitive diagnostic tools should be considered only in low transmission settings where there is already widespread implementation of malaria diagnostic testing and treatment and low parasite prevalence rates (e.g., < 10%). Use of nucleic acid amplification (NAA)-based methods should not divert resources away from malaria prevention and control interventions and strengthening of the health care services, including the surveillance system.

Suggested changes:

- A number of highly sensitive techniques are available that detect low-density infections (below 100 parasites/µl). Generally, the use of more sensitive diagnostic tools should be considered only in low transmission settings where there is already widespread implementation of malaria diagnostic testing and treatment and low parasite prevalence rates (e.g. < 10%). Use of highly sensitive methods should not divert resources away from malaria prevention and control interventions and strengthening of the health care services, including the surveillance system.

Recommendation 3 (current wording):

Submicroscopic *Plasmodium falciparum* and *P. vivax* infections are common in low as well as high transmission settings. The use of NAA methods by malaria programmes should be considered for epidemiological research and surveys aimed at mapping submicroscopic infections at low transmission intensity. There may also be a use for NAA methods for identifying foci for special intervention measures in elimination settings.

Suggested changes:

- Low-density *Plasmodium falciparum* and *P. vivax* infections are found in low as well as high transmission settings. The use of highly sensitive tests by malaria programmes ~~should~~ may be considered for epidemiological research and surveys aimed at mapping low-density infections ~~submicroscopic infections at low transmission intensity~~. There may also be a use of highly sensitive methods for identifying foci for special intervention measures in elimination settings.

Recommendation 4 (current wording):

The majority of infections with asexual parasites have gametocytes detectable by molecular amplification methods, at low density not detectable by microscopy or RDTs. Most malaria infections (microscopic and submicroscopic) should be considered as potentially infectious and able to contribute to ongoing transmission. There is no need for routine detection of gametocytes using sensitive NAA methods in malaria surveys or clinical settings.

Suggested changes:

- […] Most malaria infections (including low-density infections ~~microscopic and submicroscopic~~) should be considered as potentially infectious, ~~and able to contribute to ongoing transmission,~~ but the extent of the contribution of low-density infections to transmission has yet to be determined. There is no need for routine detection of gametocytes using highly sensitive diagnostics ~~using sensitive NAA methods~~ in malaria surveys or clinical settings.

Recommendation 5

Common standards for nucleic acid based assays should be developed, including use of the WHO International Standard for *P. falciparum* DNA NAA assays and development of standards for other Plasmodium species, particularly *P. vivax* should be undertaken. A standard operating procedure should be developed which defines methods for sample collection, extraction, and the recommended equivalent quantity of blood to be added to the assay.

Development of an international, external quality assurance system is strongly recommended to ensure that data obtained from nucleic acid amplification assays are reliable and comparable.

Suggested addition:

- […] Reports presenting NAA results should include details of the methods used for sample collection and extraction, and the equivalent quantity of blood added for the PCR reaction, as well as details of outputs in DNA copies or parasite density.

Recommendation 6

In order to establish the role of serological assays in epidemiological assessments, there is a need for standardization and validation of reagents (antigens and controls), assay methodologies and analytical approaches.

Suggested changes:

No suggested changes.

Annex 2: List of pre-reads for the meeting

Main pre-reads:

1. Slater H, Okell L. Systematic literature review on the density, temporal dynamics and infectiousness of submicroscopic *P. falciparum* infections. Unpublished.

2. Robinson LJ, Hofmann NE, Karl S. The detectability and infectivity of submicroscopic Plasmodium *vivax* infections. Unpublished.

3. Kachur P. Clinical consequences of submicroscopic *P. vivax* and *P. falciparum* malaria infections. Unpublished.

4. Slater H, Robinson LJ. Comparison between falciparum and vivax submicroscopic infections. Unpublished.

5. WHO policy recommendation on malaria diagnostics in low transmission settings. Geneva: World Health Organization; 2014.

6. WHO Evidence Review Group on malaria diagnosis in low transmission settings. Meeting report. Geneva: World Health Organization; 2014

7. Gonçalves BP, Kapulu MC, Sawa P, Guelbéogo WM, Tiono AB, Grignard L, Stone W, Hellewell J, Lanke K, Bastiaens GJH, Bradley J, Nébié I, Ngoi JM, Oriango R, Mkabili D, Nyaurah M, Midega J, Wirth D, Marsh K, Churcher TS, Bejon P, Sirima SB, Drakeley C, Bousema T. The human infectious reservoir for Plasmodium falciparum malaria in areas of differing transmission intensity. Submitted for publication.

Additional suggested pre-reads:

8. Tripura R, Peto TJ, Veugen CC, Nguon C, Davoeung C, James N, et al. Submicroscopic Plasmodium prevalence in relation to malaria incidence in 20 villages in western Cambodia. Malar J. 2017;16(1):56. doi:10.1186/s12936-017-1703-5.

9. Tripura R, Peto TJ, Chalk J, Lee SJ, Sirithiranont P, Nguon C, et al. Persistent Plasmodium falciparum and Plasmodium vivax infections in a western Cambodian population: implications for prevention, treatment and elimination strategies. Malar J. 2016;15:181. doi:10.1186/s12936-016-1224-7.

10. Imwong M, Stepniewska K, Tripura R, Peto TJ, Lwin KM, Vihokhern B, et al. Numerical distributions of parasite densities during asymptomatic malaria. J Infect Dis. 2016;213(8):1322–9. doi:10.1093/infdis/jiv596.

11. Slater HC, Ross A, Ouédraogo AL, White LJ, Nguon C, Walker PG, et al. Assessing the impact of next-generation rapid diagnostic tests on Plasmodium falciparum malaria elimination strategies. Nature. 2015;528(7580):S94–101. doi:10.1038/nature16040. PMID: 26633771

12. Nsobya SL, Parikh S, Kironde F, Lubega G, Kamya MR, Rosenthal PJ, et al. Molecular evaluation of the natural history of asymptomatic parasitemia in Ugandan children. J Infect Dis. 2004;189(12):2220–6. doi:10.1086/421281.

13. Lin JT, Ubalee R, Lon C, Balasubramanian S, Kuntawunginn W, Rahman R, et al. Microscopic Plasmodium falciparum gametocytemia and infectivity to mosquitoes in Cambodia. J Infect Dis. 2016;213(9):1491–4. doi:10.1093/infdis/jiv599.

14. Pethleart A, Prajakwong S, Suwonkerd W, Corthong B, Webber R, Curtis C. Infectious reservoir of Plasmodium infection in Mae Hong Son Province, north-west Thailand. Malar J. 2004;3:34. doi:10.1186/1475-2875-3-34.

15. Lawniczak MK, Eckhoff PA. A computational lens for sexual-stage transmission, reproduction, fitness and kinetics in Plasmodium falciparum. Malar J. 2016;15(1):487. doi:10.1186/s12936-016-1538-5.

16. Bousema T, Okell L, Felger I, Drakeley C. Asymptomatic malaria infections: detectability, transmissibility and public health relevance. Nat Rev Microbiol. 2014;12(12):833–40. doi:10.1038/nrmicro3364. PMID: 25329408

17. Bousema T, Drakeley C. Determinants of malaria transmission at the population level. In: Wirth D, Alonso P, editors. Malaria biology in the era of eradication. Long Island, NY: Cold Spring Harbor Laboratory Press; 2017.

18. Churcher TS, Bousema T, Walker M, Drakeley C, Schneider P, Ouédraogo AL, et al. Predicting mosquito infection from Plasmodium falciparum gametocyte density and estimating the reservoir of infection. Elife. 2013;2:e00626. doi:10.7554/eLife.00626. *(improved model will be available before the meeting, at least in submitted version)*

19. Lin JT, Saunders DL, Meshnick SR. The role of submicroscopic parasitemia in malaria transmission: what is the evidence? Trends Parasitol. 2014;30(4):183–90. doi:10.1016/j.pt.2014.02.004.

20. Johnston GL, Smith DL, Fidock DA. Malaria's missing number: calculating the human component of R0 by a within-host mechanistic model of Plasmodium falciparum infection and transmission. PLoS Comput Biol. 2013;9(4):e1003025. doi:10.1371/journal.pcbi.1003025.

21. Kiattibutr K, Roobsoong W, Sriwichai P, Saeseu T, Rachaphaew N, Suansomjit C, et al. Infectivity of symptomatic and asymptomatic Plasmodium vivax infections to a Southeast Asian vector, Anopheles dirus. Int J Parasitol. 2017;47(2-3):163–70. doi:10.1016/j.ijpara.2016.10.006.

22. Eisele TP, Bennett A, Silumbe K, Finn TP, Chalwe V, Kamuliwo M, et al. Short-term impact of mass drug administration with dihydroartemisinin plus piperaquine on malaria in Southern Province Zambia: a cluster-randomized controlled trial. J Infect Dis. 2016;214(12):1831–9. doi:10.1093/infdis/jiw416.

23. Chen I, Clarke SE, Gosling R, Hamainza B, Killeen G, Magill A, et al. "Asymptomatic" malaria: a chronic and debilitating infection that should be treated. PLoS Med. 2016;13(1):e1001942. doi:10.1371/journal.pmed.1001942.

24. Lindblade KA, Steinhardt L, Samuels A, Kachur SP, Slutsker L. The silent threat: asymptomatic parasitemia and malaria transmission. Expert Rev Anti Infect Ther. 2014;11:6, 623–39. doi:10.1586/eri.13.45.